ACTION SPECTRUM FOR INHIBITION OF STEM GROWTH IN DARK-GROWN SEEDLINGS OF ALBINO AND NONALBINO BARLEY (HORDEUM VULGARE)

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Introduction

Close similarity of the action spectrum for leaf elongation of dark-grown pea seedlings (*Pisum sativum*) to the action spectra for floral initiation of both short- and long-day plants is indicative of a common causative phenomenon (**7**). The reliability of this action spectrum, however, as a measure of growth of vegetative structures requires further testing, as does also the influence on it of screening by chlorophyll and carotenoid pigments of green plants. Plant material that would allow simultaneous study of these two questions accordingly was sought and was found in barley (*Hordeum vulgare*) segregating for albinism. The albino plants of the variety selected (Colsess I) appeared to be colorless whether grown in light or in dark, while the nonalbino seedlings were green when grown in light and yellow when grown in dark. Since the nonalbino seedlings were from parents heterozygous for albinism, they occurred as a mixture of pure and heterozygous types in a ratio of 1:2. The action spectrum determined was for inhibition of elongation of the second internode.

Material and methods

CULTURAL PROCEDURES.—Seven barley varieties segregating for albinism, which was known to be controlled by a single recessive factor different in each variety (**9**), were used in a preliminary experiment. Barley has seven linkage groups with a factor for albinism in any one of these except the fourth, and strains representative of each type were tested. The length of the second internode was markedly inhibited by light in every case for both albino and nonalbino, or potentially green, seedlings. Further experiments were performed with Colsess I and Nigrinudum varieties, both of which have the factor for albinism in the sixth linkage group. Colsess I proved as sensitive to radiation as any of the other strains tested, and, since more seed was initially available for increase, this variety was chosen for the action-spectrum determination. Seed was increased in each of two seasons by D. W. ROBERTSON, of the Colorado Agricultural Experiment Station, who harvested the plants as individuals. Samples of twenty-five seeds from each plant were grown in the greenhouse, and all lots in which albinos appeared were combined. Plantings from this bulked sample produced approximately 25% albinos.

Results of previous work on light-induced inhibition of elongation of the first internode of *Avena* seedlings (**5**) and of the second internode of pea seedlings (**7**) indicated a high variance inherent in the biological material. Accordingly, special efforts were made to establish and

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test conditions leading to reasonably quantitative results with barley. Seedlings for experiments were grown in white quartz sand in redwood boxes 6 cm. wide, 12 cm. deep, and 35 cm. long. Fifty dry seeds were planted in two rows 2.5 cm. apart and 2.5 cm. below the top of the box. They were covered with 2.0 cm. of sand, and a complete nutrient solution was applied daily. A flashlight equipped with filters to give very weak light restricted to the region near 5200 A was used during applications of nutrient solutions. It was established that this light had no measurable effect on the length of the second internode.

All seedlings were grown in darkrooms at controlled temperatures. In a preliminary study it was found that seedlings satisfactory for the purposes of this work could be produced at temperatures as high as 80° F. and that the time required for the complete elongation of their second internodes was much less than when they were grown at the lower temperatures required for production of normal seedlings in light. Internode elongation at 80° F. was complete in 10 days or less and at 70° in about 15 days. To reduce the time required, 80° F. was used in all subsequent experiments, since it was also found that sensitivity of the seedlings to light was as definite at this temperature as at lower ones.

Time of emergence of seedlings from the sand was somewhat variable, which suggested that individual seeds perhaps absorbed water at different rates and thus did not start germinating at the same time. Comparisons were, therefore, made of partially germinated, soaked, and dry seed. For this experiment one lot of seed was soaked 6 hours at 80° in darkness before planting, and another was soaked 6 hours and then germinated 18 hours at 80° F. A third was planted when soaking of the other two lots was started. Only seeds that appeared fully imbibed in the soaked lot and that had developed visible roots about a millimeter long in the germinated lot were used in the test. Selection and planting of seed required use of very dim light for a few minutes. The average length of second internodes of the lots from dry seed exceeded the length of those from either of the other two treatments. Whether inhibition resulted from soaking or from radiation received at the time of planting was not determined. Dry seed was planted in all subsequent tests.

Variability in length of the second internode of plants grown in darkness was observed to be so great as to require undesirably large populations to give the experiments an acceptable precision. Experiments showed that this length varied with seed weight. In subsequent work all seeds were weighed, and two classes having ranges of 31–36.5 mg. and 36.5–41.5 mg. were reserved for planting. This selection gave an increase in efficiency of 150% for seven experiments requiring about 14,000 seeds.

Rate of second-internode development for Colsess I seedlings, as shown in table 1, was followed as a guide for selection of irradiation procedure. Coleoptile tips started to emerge from the sand on the third day, and most seedlings were more than 2 cm. tall at the start of the fourth day. Therefore, lots of plants were irradiated for a single period of an hour on the fourth, fifth, sixth, or seventh day after planting. Maximum inhibition of the second internode expressed as millimeters resulted from irradiation given on the fourth day, but inhibition expressed as a percentage of subsequent growth of controls was not significantly different for irradiation on the fourth, fifth, sixth, or seventh day. Other experiments tested

the inhibitory effects of a short irradiation period on each of two successive days. This treatment gave appreciably greater inhibitions than did a single period of irradiation, and the procedure of irradiating on the fourth and fifth days after planting and of harvesting on the tenth was adopted. Separate records were made for albino and potentially green seedlings.

Continuous irradiation was also tested and resulted in maximum inhibitions leading to second internodes less than 3 mm. in length. This procedure, however, was not necessarily desirable and was not practical, since, with the equipment available for action-spectrum determinations, adequate populations could not have been readily obtained.

IRRADIATION PROCEDURES.—The objective was to measure the energy required for a given inhibition of the second internode of barley at various wave lengths. This was realized by determining the internode length resulting from irradiation for a short time in each of several wave-length regions. Reduction of these data required estimation of the energies that would have been necessary at each station to produce equal internode length. Such estimates were based on dosage experiments that showed the variation of internode length with energy and on a reciprocity determination which established that a given energy applied at different intensities within the limits used in these experiments resulted in identical response. Reduction of data thus followed the previous work on enhancement of leaf length for dark-grown pea seedlings (**7**).

Radiation from an incandescent-filament lamp was used for the dosage and reciprocity experiments. This is approximately equivalent to use of radiation between about 6000 A and 7000 A, which is the most effective portion of the spectrum. Boxes of seedlings were irradiated at stations at various distances from the source; these distances were established with a barrier layer photocell to give the desired range of intensities. In the dosage experiments energies were varied by about ten thousand fold, and in the reciprocity experiments times of irradiation were varied by one hundred fold.

TABLE 1

LENGTH ATTAINED BY SECOND INTERNODES OF POTENTIALLY GREEN COLSESS I BARLEY SEEDLINGS AFTER VARIOUS DAYS OF GROWTH IN DARKNESS AT 80° F

DAYS AFTER PLANTING	LENGTH OF INTERNODE (MM.)		
	Expt. 17	Expt. 18	Expt. 22
3	2.0	.....	.....
4	3.4	2.6	3.1
5	5.7	4.2	4.0
6	18.7	7.5	9.2
7	33.2	18.8	23.3
8	42.3	.....	.....
9	62.7	.....	.....
10	63.9	55.9	56.7

In spectrographic experiments use was made of the apparatus described in previous work on the action spectrum for control of floral initiation by long- and short-day plants (**6**). Boxes containing seedlings were brought from the growth rooms on the fourth and fifth days after planting and placed at previously determined positions in the focal plane of the spectrograph. All these transfers were made in darkness. Wave-length positions at distances of 5 cm. along the boxes were previously established by reference to the emission spectrum of a carbon loaded with barium oxide. After irradiation the boxes were returned to darkness. Other lots were then irradiated until the desired population had been attained. All plants in each 5-cm. interval were

harvested separately and measured. The thermopile used for measuring energies incident on the plants at various wave lengths was calibrated as previously described (6) against radiation from a standard carbon-filament lamp certified by the National Bureau of Standards. The slit width and focus of the spectrograph were different for experiments in the blue and red portions of the spectrum, the effective slit width being about 100 A at 6500 A and 4800 A.

TABLE 2

VARIATION OF SECOND-INTERNODE LENGTH OF BARLEY WITH INTENSITY OF CONSTANT ENERGY (RECIPROCITY)

INTENSITY (FT.-C.)	TIME (MIN.)	LENGTH OF SECOND INTERNODE*	
		Potentially green	Albino
25.0.....	1	49.2	69.7
2.5.....	10	47.5	66.8
0.25.....	100	49.0	73.6

* Coefficient of variability: potentially green, 7.04; albino 8.95. No significant differences at 19:1.

Results

Internode lengths were not found to be significantly different for treatments with equal energy in which intensity was varied from 0.25 to 25 foot-candles and time from 100 minutes to 1 minute; i.e., reciprocity held over this range of intensities and times (table 2). Actual inhibitions in length of the second internode were about 20 mm. for both albino and potentially green plants with populations of about 30 and 120, respectively, at each station.

In four experiments that covered a range of energies from 0.03 to 150.0 foot-candle minutes (ft.-c.m.) the inhibition varied linearly with the logarithm of the energy (fig. 1). These inhibitions were obtained by subtracting observed internode lengths from mean internode lengths of nonirradiated control lots of many experiments. Internode lengths of controls were 69 mm. for potentially green plants and 89 mm. for albinos. Each point on the curve for the potentially green plants was derived from a population of about 110 and, for albinos, of about 35. In the four experiments the linear, quadratic, and cubic components of the regression of inhibition on log energy were calculated, and the linear terms were found to account for 93.7–99.6% of the variation in inhibition values. Variance analysis for the combined data for potentially green and albino seedlings indicated that the two did not differ significantly in linearity.

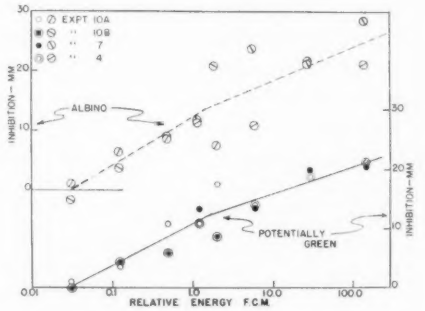

FIG. 1.—Variation in inhibition in length of second internode of dark-grown Colsess I barley seedlings with radiant energy. Population of albinos about 36; of potentially green about 110. Results from four experiments are shown by various symbols.

Two experiments, 3 and 8, were performed in the red portion of the spectrum. Internode lengths observed at various wave-length stations for experiment 8 are shown in table 3, along with the necessary steps to convert the actual energies to relative energies required for a given arbitrarily selected response. The

observed internode lengths ranged from 53.7 to 57.2 mm. and, on the basis of a 69-mm. control, corresponded to inhibitions of 11.8 to 15.3 mm., which fell on the upper part of the curve in figure 1. An internode length of 54 mm. which was within this range was selected as an arbitrary reference for converting the actual energies to relative energies for a given response. At 6870 A the observed internode length at harvest was 53.7 mm. after irradiations of 90 seconds with 77.4 kiloergs/sq cm on both the fourth and the fifth days. To reduce the actual energy of 77.4 kiloergs/sq cm to an energy which would have given a 54-mm. internode length, the actual energy had to be multiplied by the factor 0.87. This factor was derived from figure 1 by selecting any inhibition on the upper part of the curve and noting the energies corresponding to this inhibition and to an inhibition 0.3 mm. less than this value. The first value divided by the second gave the conversion factor. This factor of 0.87 multiplied by 77.4 thus gave the required energy of 67 kiloergs/sq cm, which was an absolute value for the particular experiment.

Energy values at a given wave-length station, 6870 A, for instance, were different for the two experiments, and the differences varied from station to station. The differences were affected not only by variation within a particular experiment but also by any variation between the two experiments. Responses at stations

TABLE 3

EXPERIMENTAL DATA AND THEIR REDUCTION FOR POTENTIALLY GREEN BARLEY SEEDLINGS OF EXPERIMENT 8

WAVE LENGTH AT CENTER OF STATION, A	POPULATION	AV. LENGTH OF SECOND INTERNODE, MM.	CONVERSION FACTOR TO 54.0 MM.	ENERGY KILOERGS/SQ CM		RELATIVE ENERGY FOR A GIVEN RESPONSE
				Actual	Referred to 54.0 mm.	
6870	84	53.7	0.87	77.4	67	0.55
6630	88	55.2	1.85	72.0	133	1.08
6430	93	55.1	1.78	66.6	119	0.97
6240	89	54.9	1.61	63.0	101	0.82
6060	99	56.6	3.89	59.4	231	1.89
5900	93	57.2	5.10	55.8	285	2.33

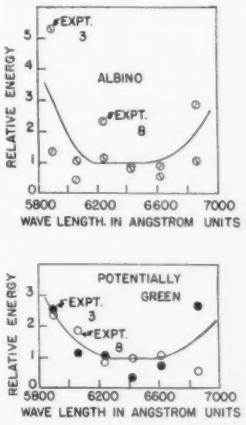

FIG. 2.—Action spectra for inhibition of growth of second internode of Colsess I barley seedlings by red portion of spectrum. Populations approximately 100 and 175 for potentially green seedlings in experiments 8 and 3, respectively; 35 and 60 for albino seedlings.

6630, 6240, and 6060 A were similar in both experiments; so the mean of the responses at these stations was taken as corresponding to a relative energy of 1.0. Relative energies obtained in this way

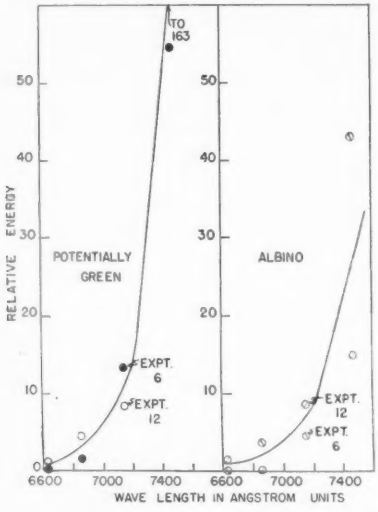

FIG. 3.—Action spectra for inhibition of second internodes of potentially green and albino Colsess I barley seedlings by wave lengths greater than 6600 A.

are given in the last column of table 3, and results from both experiments are shown in figure 2. Data on albino seedlings were treated similarly, and results obtained are also shown in figure 2.

Two experiments, 6 and 12, extended the action curve beyond 7000 A (fig. 3) and covered the region of rapid change in effectiveness between 6000 and 5300 A (fig. 4). The populations of potentially green plants at each station were about seventy and eighty-five, respectively. Two stations at each end of the curve of figure 2 duplicated stations in experiments 6 and 12, and these served for establishing the relative response scale normalized to that of figure 2. When the results of experiments 6 and 12 were thus normalized to those of experiments 3 and 8, the agreement was satisfactory both in the region of 5800–6100 A and in the region of 6600–6850 A. At 7400 A (fig. 3) the energy requirement for a given response was approximately fifty times that at 6600 A. Stations at 7820 A and at about 8300 A were also used in addition to those shown, and the energy requirements at these wave-length regions were found to be at least one hundred times the requirement at 6600 A. In going from 6000 A to 5200 A, experiments 6 and 12 (fig. 4), there was also a sharp increase in energy requirement for potentially green seedlings. Thus at 5400 A the requirement was about twenty times that at 6000 A.

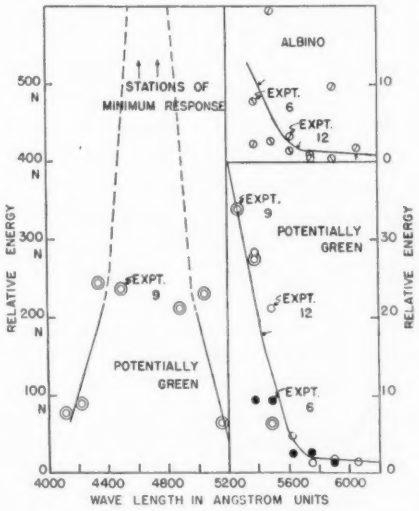

FIG. 4.—Action spectra for inhibition of second internode of Colsess I barley seedlings by wave lengths less than 6100 A.

The data for albinos were reduced as for the potentially green, but the agreement with the experiments in the red portion of the spectrum (fig. 3) was less precise. This lack of agreement probably resulted for the greater part from the fact

that albino populations averaged only twenty-three and twenty-eight for experiments 6 and 12, respectively. Despite the variability, it was evident that the response of albino seedlings was essentially the same as that of potentially green seedlings. The curve for albinos at the blue end of the spectrum (fig. 4) was drawn through the average value of four points at 5380 A and 5490 A and of four others at 5620 A and 5750 A. These values are indicated by arrows on the curves, as is also the normalized value of 1.0 at 6060 A. Here also the albino response was found to be basically the same as that of the potentially green.

The action curve from 4085 to 5495 A, which included the region of minimum response in the visible part of the spectrum, was determined in experiment 9. Some of the data of experiment 9 plotted in figure 4 were obtained by combining data from several stations as indicated in table 4. The curve for potentially green plants of experiment 9 was normalized to that of experiments 6 and 12. To do this, the four values for wave-length stations 5380 and 5495 A of experiments 6 and 12 were averaged, and the curve was drawn through this point, indicated by an arrow on the curve. The values of experiment 9 for these same wave-length stations were also averaged and normalized to experiments 6 and 12 through this point.

It was necessary to use 40-minute irradiation periods in experiment 9, and even then only small effects were obtained in the region of the minimum response, that is, the region in which maximum energy was required for a response equal to that produced in the red. In previous experiments the inhibitions were all greater than 12 mm., and thus only the upper part of the curve of figure 1 was necessary for conversion of the data. Absolute values of the inhibitions were, therefore, not required to obtain relative energies of a given effect throughout such experiments. In experiment 9, however, the inhibitions were considerably less, necessitating use of both ends of the curve of figure 1 for converting the observed energies to those required for uniform response. In this instance absolute rather than relative inhibition values were necessary to determine which points fell on one slope of the inhibition curve and which on the other. Absolute inhibitions were subject to greater error than relative values, however, since the control samples by means of which the inhibitions were estimated were also subject to random variation. For these reasons and because the response curve was normalized to that in the green, which in turn had been normalized to that in the red, considerable uncertainty existed about the relative response scale. Ordinates are accordingly given as 100 N, 200 N, etc., in figure 4, where N is a multiplier expressing these uncertainties. The value of N is probably between 0.25 and 1.00. These limits gave a minimum sensitivity between 1/250 and 1/1000 of the maximum sensitivity in the region of 6500 A. In any case the position of the minimum response is known to be in the region of 4590 A–4770 A.

Results for albino plants, also shown in table 4, were even more irregular because of the small populations. They followed the general behavior of the potentially green plants, however, with a minimum of response in the same general region.

Discussion

The action spectrum for inhibition of growth in length of the second internode of dark-grown barley seedlings corresponds closely to that for stimulation of leaf growth in dark-grown pea seedlings

(7). In both cases there is some uncertainty about the spectral region of minimum sensitivity. Although this was indicated as being near 4900 A for pea seedlings, it can really only be said to be in the region of 4600–4900 A. Both action spectra closely resemble those found for control of floral initiation in short-day plants such as soybean and cocklebur and in long-day plants such as barley and *Hyoscyamus niger* (8). The conclusion seems to be warranted that these very different responses arise from the same causative phenomenon. No postulate has been advanced as to the nature of the initial action of light, and no experiments have been brought to bear on the subject.

Two pertinent questions have not yet been raised here about the inhibition of the length of the second internode. The first of these relates to the nature of the inhibition: Is it cell division or cell elon-

TABLE 4

EXPERIMENTAL DATA ON INHIBITION OF SECOND-INTERNODE LENGTH OF COLSESS I BARLEY BY RADIATION IN REGION OF 4085–5495 A

Station	Wave length, A	Energy kiloergs/sq cm	Av. length of potentially green internodes, mm.*	Inhibition of potentially green internodes, mm.†	Av. length of albino internodes, mm.*	Inhibition of albino internodes, mm.†
1	5495	2,880	49.7	17.3	76.4	13.3
2	5380	2,740	52.7	14.3	73.0 (75.7)	
3	5270	2,570	53.2	13.8	79.5	10.2
4	5170	2,450	54.6	12.4	78.2 (78.8)	
5	5080	2,230	57.0	8.2	85.5	1.0
6	5000	2,020	60.7 (58.8)		90.4 (88.0)	
7	4920	1,900	58.8	8.1	77.8	11.0
8	4845	1,780	59.0 (58.9)		78.2 (78.0)	
9	4770	1,630	64.2	3.0	78.4	5.0
10	4700	1,460	63.8 (64.0)		89.7 (84.0)	
11	4640	1,320	62.4	3.1	91.4	1.6
12	4590	1,200	65.4 (63.9)		89.9 (90.6)	
13	4535	1,030	61.2	5.6	78.5	9.7
14	4485	890	60.2 (61.4)		79.3 (79.3)	
15	4435	790	62.9		80.1	
16	4385	700	65.7	4.6	87.7	5.0
17	4340	670	61.7 (62.4)		79.6 (84.0)	
18	4300	620	59.9		84.6	
19	4260	600	58.7	7.1	79.0	7.1
20	4220	560	61.0 (59.9)		83.2 (81.9)	
21	4180	540	59.9		83.4	
22	4150	520	63.4	7.1	80.0	5.7
23	4115	500	60.0 (59.9)		83.9 (83.3)	
24	4085	480	56.2		85.9	

* Mean populations per station: potentially green, 50; albino, 17.

† Based on lengths of 67.0 mm. and 89.0 mm., respectively, for internodes of potentially green and albino control seedlings grown in darkness.

gation or both? The second is: What is the region of light action in the plant? The first question has been investigated for *Avena* by a number of workers (1, 3, 5, 10), with varying results. AVERY (1) and GOODWIN (5) each called attention to the fact that both processes were involved at high energies of effective radiation, but that at low energies only cell division was affected. In their experiments the ranges of low energies were of the order of magnitude of the highest energies used in this work. No attempt was made, however, to differentiate between these two possible effects of light on barley seedlings. GOODWIN found that the red portion of the spectrum was most effective for inhibition of the length of the first internode of *Avena*. The same region of the spectrum was found to be most effective for inhibition of the second internode of barley.

To answer the second question, Colsess I barley seedlings were irradiated on the sixth day when their first leaves had elongated considerably beyond the coleoptiles. When radiation was applied only to these extended tips, the internodes were markedly inhibited. This experiment showed that direct irradiation of the internode was not necessary but did not exclude the internode as also being a potential receptor. When seedlings were irradiated in the customary manner on the fourth and fifth days, the coleoptile which was directly irradiated scattered light to the inclosed leaves. The internode, which was about 1.5 cm. below the surface of the quartz sand, could have received only a very small amount of scattered radiation.

In the barley varieties tested it is apparent that chlorophyll synthesis was disrupted in the albinos. In the synthesis of chlorophyll this disruption might take place at protochlorophyll or earlier, but it would not be expected to prevent synthesis of protoporphyrin IX, which supposedly is required for synthesis of hemin containing enzymes such as catalase and peroxidases. Mr. C. E. HAGEN very kindly tested the activity of these two enzymes in light- and dark-grown albino and nonalbino Colsess I seedlings. Catalase activity was three to four times as great in albino as in nonalbino seedlings, while peroxidase activity was reversed. The significant point, however, was that each enzyme was present in considerable amounts in every lot as was found for albino corn seedlings by EYSTER (4).

An open-chain tetrapyrolle of the phycocyanin type could either be a precursor of protoporphyrin IX, derived from it, or made quite independently from more primitive precursors. The action spectra indicate a chromophoric group of this type in the effective pigment. If the first of the possibilities mentioned is the case, then absence of the open-chain tetrapyrolle would probably be lethal for all plants.

The results obtained for albino barley seedlings, together with results for photoperiodic response of the several plants that have been studied, indicate that possible screening pigments do not seriously modify the action spectra. The variation in the relative absorption coefficients for the several plants was not more than tenfold in any of the wave-length regions studied. The ratio of the energy required for a given response at the least effective part of the visible spectrum to that at the most effective part is smallest for prevention of floral initiation in soybean (6). Since this was the first species examined, it seemed best to repeat the measurement of this ratio. It was found to be the same as previously reported within the limit of experimental error, which is possibly as great as twofold. The effective pigment,

if present in cells of the mesophyll, as seems quite likely, would apparently have to be exterior to the plastids.

The effective pigment still remains undetected by visual or isolative methods applied to plants. A direct test for an absorption in the red portion of the spectrum was made on tissue of albino plants to place an upper limit on this absorption. Clippings of dark-grown albino seedling leaves that had been held below 5° F. for about 9 months were infiltrated with glycerine under vacuum to reduce light scattering. This infiltrated tissue was then packed into a 1-cm. thick cell of a Beckman spectrophotometer and its transmission measured from 7750 A to 5000 A. Less than 0.1% of the incident light was characteristically absorbed in the region of 6400 A. Even this upper limit is at least one hundred fold greater than would be absorbed characteristically by a single albino seedling; accordingly, not more than one part in one hundred thousand of the incident light is utilized even in the region of maximum effectiveness, and this falls by a hundred to a thousand fold in the region of minimum effectiveness. Although this first experiment assures that the pigment is not self-screening, it is discouraging for direct detection. Albino seedlings, nevertheless, offer attractive material for attempted separations, since they would eliminate the considerable confusion of chlorophyll following through possible steps in protein fractionation.

More than a thousand-fold change in energy is required to pass from a small to a large inhibition of the second internode length of barley (fig. 1). In the case of floral initiation this same order of change is brought about by less than a tenfold change in energy. This latter degree of responsiveness is characteristic for many simple reactions, such as enzymatic inhibitions, as a function of inhibitor concentration.

Floral initiation in most long-day plants is also accompanied by a change in elongation of upper internodes. The change, however, is an enhancement rather than an inhibition by light. In the case of barley (2) it is coupled closely with control of floral initiation and would be said to have the same action spectrum. It, too, shares the characteristic of passing through most of the region of response with less than a tenfold change in energy. Finally, although the degree to which cell elongation and cell division contribute to this internode elongation in barley has not been determined, cell elongation surely plays a prominent part.

Equivalence in the initial light reaction for inhibition of the second internode length in barley and for control of floral induction in long- and short-day plants is supported by reciprocity experiments. Radiation is strictly additive in its effects in both cases if given over periods covering an appreciable fraction of a normal night. This indicates that the action of light starts processes that proceed undiminished for the course of several hours. It reflects the fact that a dark period, in the case of photoperiodism, must have a critical length in order for reactions to proceed that are limited by irradiation at the end of the light period. In the case of internode elongation in dark-grown seedlings this dark-period length is equivalent to the period required for considerable deviation from additivity in the effects of two irradiations separated by a time period.

Summary

1. Growth in length of the second internode of dark-grown barley seedlings can be inhibited by a relatively small

amount of radiant energy given at one or more times.

2. Both potentially green and albino seedlings of barley were sensitive to radiation, and in the variety Colsess I, which was studied in detail, they were approximately equal in sensitivity.

3. The action spectra for control of growth of the second internode of dark-grown potentially green and albino Colsess I barley seedlings were quantitatively determined but with somewhat less precision for the albino than for the potentially green seedlings.

4. The two action spectra were the same within the limits of error as those previously found for control of leaf length in dark-grown pea seedlings and for control of floral induction in both long- and short-day plants. This indicated that the phenomena depended upon the same initial photoreaction.

5. More than a thousand-fold change in energy is required to pass from a small to a large inhibition of the second-internode length of barley, but, in the case of floral initiation, this same order of change is brought about by less than a tenfold change in energy.

6. The effective pigment absorbed less than one part in one hundred thousand of the radiation incident on an albino barley seedling even in the region of its maximum absorption in the red portion of the spectrum.

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ALCOHOL-INSOLUBLE SOLIDS OF JUICE VESICLES AND PULP OF CITRUS FRUIT[1]

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Introduction

Although the soluble constituents of the juice vesicles and pulp of citrus fruits have been determined and studied in considerable detail (1, 13), the insoluble solids have not been subject to such investigations. In comparison with the soluble constituents, the insoluble solids of the vesicles and pulp are present in the fruit in relatively low concentrations; this fraction is nevertheless important in the physiology of the juice vesicles. The insoluble solids, which include the cell-wall constituents such as cellulose, lignin, pectin, and hemicellulose, are designated as polysaccharides or acid-hydrolyzable materials. These substances, especially pectin and hemicellulose, appear to be closely related to one another.

The present investigation is concerned chiefly with the determination of the various constituents in the alcohol-insoluble solids of the juice vesicles of Valencia and navel oranges and of the pulp of navel oranges and lemons. Certain relationships are also noted between the pectins and other constituents of the alcohol-insoluble solids.

Material and methods

Samples of fifty to one hundred fruits each of mature navel and Valencia oranges and of lemons were taken from plots of trees located in experimental orchards at the University of California Citrus Experiment Station and in commercial groves in the vicinity of Riverside, California. The fruits were peeled, and segments were separated. Juice vesicles were easily removed from the segments by cutting away the segment walls with a sharp knife and were finely ground to a fluid state in a Waring Blendor. Aliquot portions of this material (1000–1500 ml.) were poured into five times their volume of 80% alcohol, a correction being made for the moisture in the sample. The alcohol-insoluble solids settled to the bottom of the container. The solution was stirred at intervals, allowed to stand overnight, and finally filtered on a Büchner funnel. The residue was extracted a second time with 7 liters of 95% alcohol. This mixture was stirred frequently, allowed to stand overnight, filtered on a Büchner funnel, and washed thoroughly with 95% alcohol. The material was dried in a ventilating oven at 65° C. and then ground to a fine powder in a Wiley mill. The resulting product was a light, white, somewhat fibrous substance, the alcohol-insoluble solids, on which the analyses were made.

For moisture determinations, samples of vesicles and pulp were removed from the fruit. Other samples were weighed and used for quantitative determination of the alcohol-insoluble and alcohol-soluble solids. A much larger portion was used for obtaining a large experimental sample of alcohol-insoluble material.

For quantitative determinations, the fresh material was finely ground to a fluid state in a Waring Blendor in the presence

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of 95% ethyl alcohol. These samples were immediately heated to boiling temperature and allowed to stand overnight in 300 ml. of 80% ethyl alcohol, a correction being made for the moisture in the sample. Two additional extractions were made with hot 80% ethyl alcohol. For each extraction the samples remained in the extractant for 24 hours. The insoluble material was filtered off and washed thoroughly, first with 95% ethyl alcohol and finally with petroleum ether. The colorless product was dried to constant weight in an oven at 65° C. and weighed quantitatively. The percentages of alcohol-insoluble solids were calculated from this weight.

Pectin was extracted from the alcohol-insoluble solids by placing 0.5 gm. of the material in a 150-ml. beaker and adding 50 ml. of hot water. The beaker was placed on an asbestos gauze over a low flame, and the mixture was heated gently to slow boiling for a 10-minute extraction period. While hot, the extract was filtered with suction through a hardened filter paper (11-cm., Whatman no. 50). The solid material was scraped from the paper and returned to the beaker with 50 ml. of boiling water, and the process was repeated. A third extraction was made in the same way, all the filtrates being received in the same flask. The combined solution was filtered by suction through a small, soft filter paper (Whatman no. 42), and the filter was washed with hot water. The extract was transferred to a 600-ml. beaker and diluted to 300-ml. volume.

Pectin in the aqueous extract was determined as calcium pectate by the method of CARRÉ and HAYNES (3), with slight modifications. The pectin was saponified by adding 6 ml. of 5 *N* sodium hydroxide to the 300-ml. aqueous extract, and the solution was allowed to stand for 1 hour. Fifty milliliters of *N* acetic acid were added, with stirring, and, after 5 minutes, 50 ml. of *M* calcium chloride were added and stirred. The solution was allowed to stand 1 hour or longer for the complete precipitation of calcium pectate. The precipitate was filtered by suction through a hardened filter paper (11-cm., Whatman no. 50) on a Büchner funnel. When the volume in the funnel had been reduced to about 50–75 ml., the precipitate was washed with 200–250 ml. of boiling water. The washing was continued until the filtrate was free of chlorides. (The volume of material in the funnel must not be less than 50 ml. at any time; otherwise, filtering becomes too slow and washing difficult.) When washing was complete, the precipitate was sucked dry enough so that it would adhere to the paper when it was lifted from the funnel. The matched paper was then wetted with distilled water, and both papers were placed in an oven and dried to constant weight at 105° C., using the balanced paper as tare. The dried calcium pectate loosens from the paper and is readily removed as a thin film for analysis.

The material from the alcohol-insoluble solids remaining after three extractions with boiling water was then extracted in a similar manner with three successive 50-ml. portions of hot *N*/75 hydrochloric acid (pH 2.5). The combined solutions were filtered and saponified by adding NaOH. Sufficient concentrated hydrochloric acid was added to the extract, with stirring, to make a 10% solution. This precipitated pectic acid in a form which could be filtered readily, with suction, through a hardened filter paper. Without washing, the paper and precipitate were returned to the beaker. About 100 ml. of hot water were added, together with one drop of phenolphthalein solution and enough

ammonium hydroxide to produce a faint pink. The pectic acid readily dissolved. The paper was returned to the funnel, and the solution was drawn through it two or three times to dissolve any precipitate adhering to the funnel. Finally, it was washed with hot water. The total volume was again about 300 ml. Calcium pectate was precipitated and treated as in the aqueous extract.

The residue remaining after the hydrochloric acid extraction was washed with hot water, transferred to a weighed watch glass, and dried at 105° C. This residue was designated as the cellulose and hemicellulose fraction.

Uronic acids of the samples were estimated by determining carbon dioxide by the method of LEFEVRE and TOLLENS (8), as modified by DICKSON *et al.* (4).

Methoxyl groups were determined by the saponification procedure of ROMEO (11). Values obtained by this method compare favorably with those obtained by the ZEISEL method (16, 17).

Furfural determinations were made by the colorimetric procedure described by BRYANT *et al.* (2).

Calcium was determined volumetrically by treating the oxalate with dilute sulfuric acid and subsequently titrating the liberated oxalic acid with standard potassium permanganate.

Results

ALCOHOL-INSOLUBLE FRACTION OF VESICLES AND PULP.—The vesicles and pulp of citrus fruits (table 1) contain less alcohol-insoluble substances than the peel (14, 15). In Valencia and navel orange vesicles the alcohol-insoluble solids averaged, respectively, 0.93 and 1.40% of the fresh weights of the tissue. The mean value for lemon pulp was 1.40%. On a fresh-weight basis, the vesicles of navel oranges contained greater quantities of alcohol-insoluble materials than those of Valencia oranges.

The soluble constituents, such as sugars, organic acids, most of the inorganic compounds, amino acids, essential oils, and glucosides, were extracted from the vesicles and pulp with 80% alcohol. The alcohol-insoluble fraction, which is composed chiefly of cell-wall constituents, accounted for a relatively small part of the total dry matter. It is evident that most of the dry matter of the pulp is soluble in 80% alcohol.

CARBON DIOXIDE CONTENT OF ALCOHOL-INSOLUBLE SOLIDS.—The amounts of CO_2 produced on hydrolyzing the different samples with 12% HCl are a measure of their total uronide contents. The alcohol-insoluble fractions of Valencia orange vesicles (table 1) yielded more CO_2 (mean, 6.02%) than those of navel orange vesicles (mean, 5.50%). The alcohol-insoluble fraction of lemon pulp yielded, on the average, 8.47% CO_2, which is approximately the same amount obtained from similar fractions of lemon peel (14). The different amounts of CO_2 in the various alcohol-insoluble fractions are directly related to different amounts of galacturonic acid anhydride, as the latter values were calculated by multiplying the percentages of CO_2 by 4.

The degree of esterification of the carboxyl groups is shown by the amounts of methoxyl reported as percentages of the alcohol-insoluble solids (table 1). The fraction from Valencia orange vesicles contained the lowest percentage of methoxyl, and that from lemon pulp, the highest. The fraction from the whole pulp of the navel orange was higher in methoxyl than that from the juice vesicles only.

The total CO_2 of a given fraction is equal to the sum of the esterified and non-esterified carboxyl groups, expressed

as CO_2 (table 1). The non-esterified groups represent the carboxyl groups combined with the inorganic cations. The relatively low methoxyl content and the absence of free acidity indicate that some of the carboxyl groups are combined with the cations of the alcohol-insoluble fraction. This can be shown experimentally by converting the methoxyl values to equivalent percentages of CO_2, plus the CO_2 calculated from the free acidity of the ash-free alcohol-insoluble solids. For example, the CO_2 equivalent to 2.83% methoxyl (mean value for Valencia vesicles) is 4.02%, while that equivalent to the free acidity of the ash-free alcohol-insoluble solids is 2.06%, and the sum of these (6.08%) is the total CO_2. This value is in good agreement with the 6.02% CO_2 determined on the alcohol-insoluble solids by hydrolysis with 12% HCl. It follows from these results that the galacturonic acid anhydride values calculated from the total carboxyl groups should be equal to the values calculated from the percentages of CO_2 in the alcohol-insoluble solids. The two sets of figures show close agreement.

TABLE 1

CARBON DIOXIDE AND METHOXYL CONTENTS OF ALCOHOL-INSOLUBLE SOLIDS (A.I.S.) OF VESICLES AND PULP OF CITRUS FRUIT IN RELATION TO THEIR EQUIVALENTS OF GALACTURONIC ACID ANHYDRIDE

Sample no.	Moisture (% fresh wt.)	Alcohol-insoluble solids		CO_2 produced on hydrolysis with 12% HCl		% methoxyl in A.I.S.*	Galacturonic anhydride		Carboxyl groups (as CO_2)			Non-esterified —COOH in A.I.S. (me/gm)	Calcium in A.I.S. (me/gm)	Alkaline ash in A.I.S. (me/gm)
		% dry weight	% fresh weight	% dry weight	% A.I.S.*		$CO_2 \times \frac{3}{4}$ (% A.I.S.)*	Equivalent to total carboxyl group (% A.I.S.)*	Non-esterified (% A.I.S.)*	Esterified (% A.I.S.)*	Total (% A.I.S.)*			
Valencia orange vesicles														
1	90.39	9.36	0.90	0.57	6.08	2.79	24.32	23.88	2.01	3.96	5.97	0.46	0.52	0.61
2	90.14	9.87	.96	.61	6.15	2.77	24.60	24.40	2.17	3.93	6.10	.40	.60	.60
3	90.04	10.38	.94	.60	5.82	2.94	23.28	24.72	2.01	4.17	6.18	.46	.59	.87
Mean	90.40	9.87	0.93	0.59	6.02	2.83	24.07	24.33	2.06	4.02	6.08	0.46	0.57	0.69
Naval orange vesicles														
1	90.69	15.25	1.42	0.84	5.50	3.10	22.00	22.16	1.14	4.40	5.54	0.26	0.52	0.72
2	90.89	15.26	1.39	.82	5.38	3.15	21.52	22.28	1.10	4.47	5.57	.25	.58	.76
3	90.76	14.93	1.38	.84	5.61	3.05	22.44	22.00	1.17	4.33	5.50	.27	.51	.60
Mean	90.78	15.15	1.40	0.83	5.50	3.10	21.99	22.15	1.14	4.40	5.54	0.26	0.54	0.69
Navel orange pulp														
1	87.77	15.78	1.93	1.03	6.54	3.97	26.16	27.56	1.26	5.63	6.89	0.29	0.70	0.76
2	87.70	15.20	1.87	1.05	6.89	3.97	27.56	28.24	1.43	5.63	7.06	.32	.69	.74
3	87.77	15.70	1.92	1.06	6.74	3.92	26.96	27.96	1.43	5.56	6.99	.32	.69	.75
Mean	87.75	15.56	1.91	1.05	6.72	3.95	26.89	27.92	1.37	5.61	6.98	0.31	0.69	0.75
Lemon pulp														
1	89.03	13.74	1.48	1.15	8.40	4.71	33.60	34.56	1.96	6.68	8.64	0.45	0.35	0.53
2	90.03	13.11	1.31	1.12	8.55	4.05	34.20	34.32	1.98	6.60	8.58	.45	.30	.53
3	89.86	14.03	1.40	1.19	8.45	4.76	33.80	34.80	1.94	6.76	8.70	.44	.36	.53
Mean	89.94	13.63	1.40	1.15	8.47	4.71	33.87	34.56	1.96	6.68	8.64	0.45	0.36	0.53

* Calculated on moisture-free basis.

The alkaline ash is a measure of the cations combined with organic substances in the alcohol-insoluble fractions (table 1). The substances in combination with the mineral constituents are chiefly pectin and a smaller amount of organic acids that are insoluble in 80% alcohol. As the alcohol-insoluble fraction contains considerable pectin, it is reasonable to expect the mineral constituents to be combined with the non-esterified carboxyl groups. The difference between the alkaline ash and the non-esterified groups is equal to the minerals combined with organic substances other than pectin. In all samples except those of lemon pulp, the non-esterified carboxyl groups (milliequivalents per gram) were less than the calcium contents. This condition indicates that a small portion of the calcium was combined with an organic substance other than pectin. The high acidity or low pH of the lemon pulp is probably associated with the lower calcium content of the alcohol-insoluble fraction.

The data of table 2 show the relation among the methoxyl, galacturonic acid, and carboxyl groups of the alcohol-insoluble fractions of the different samples of vesicles and pulp. The galacturonic acid values were determined by multiplying the percentages of CO_2 by 4.411. In addition to the methoxyl values experimentally determined on the alcohol-insoluble fraction, the methoxyl values equivalent to the total carboxyl groups are also recorded. The degree of esterification of the galacturonic acid in the alcohol-insoluble fractions is shown by the ratio of methoxyl to galacturonic acid. This ratio varies somewhat with the pulp samples, it being lowest (mean, 0.107) in the Valencia orange vesicles, and highest (mean, 0.134) in navel orange pulp. The ratios of the navel orange vesicles (mean, 0.128) and of the lemon pulp (mean, 0.126) fell between these two extremes.

In terms of equivalent methoxyl, the ratios of total carboxyl groups to galacturonic acids show the greatest amount of methoxyl that can occur in the polygalacturonide of the alcohol-insoluble fractions. The mean ratios ranged from 0.161 for both Valencia and navel orange vesicles to 0.166 for navel orange pulp. These values agree satisfactorily with the theoretical values reported for a completely esterified polygalacturonide (**7**). The close agreement between theoretical and experimental values is evidence that the total carboxyl groups are derived from the pectin fraction of the alcohol-insoluble solids.

The percentages of the total carboxyl groups esterified are reported in the last column of table 2. Esterification was lowest (mean, 66.09%) in Valencia orange vesicles and highest (mean, 80.47%) in navel orange pulp.

RELATION OF URONIC ACID ANHYDRIDE TO FURFURAL CONTENT OF ALCOHOL-INSOLUBLE SOLIDS.—The yield of furfural from citrus pulp is derived chiefly from the pentoses of the pentosans and from the uronic acids and the pentoses of the pectins. In plant materials the uronic acids occur chiefly in the pectins and the hemicelluloses; in citrus pulp, however, the total uronic acid content is highly and positively correlated with the total amount of pectin isolated. The furfural obtained by the difference between the total furfural of the vesicles and pulp and the amount equivalent to the uronic acids is ordinarily used to calculate the pentosans. On hydrolysis with 12% HCl, uronic acids yield a quantitative amount of CO_2, but the furfural produced is less than the theoretical amount.

LEFEVRE and TOLLENS (**8**) found that glucuronic acid anhydride, on hydrolysis

with 12% HCl, yielded, on the average, 19.11% of its weight as furfural, or a ratio of furfural to glucuronic acid anhydride of 1:5.23. Similar determinations were made by NORRIS and RESCH (10), who found that glucuronic acid anhydride (euxanthic acid) yielded 21.48% of its weight as furfural, or a ratio of furfural to glucuronic acid anhydride of 1:4.66. With galacturonic acid anhydride, the yield of furfural (10) was 23.50%, or a ratio of furfural to galacturonic acid anhydride of 1:4.26. The percentage of furfural derived from galacturonic acid anhydride is equal to the latter divided by 4.26. This relationship gives a close approximation of the amount of furfural yielded by the uronic acids of the alcohol-insoluble solids. Substances other than uronic acids and polyuronides that yield furfural, such as pentoses and hexoses, are not present in

TABLE 2

RELATIONS BETWEEN METHOXYL, GALACTURONIC ACID, AND CARBOXYL GROUPS OF ALCOHOL-INSOLUBLE SOLIDS (A.I.S.)

SAMPLE NO.	TOTAL CO_2 (% A.I.S.)	% GALACTURONIC ACID IN A.I.S. (CO_2 ×4.411)	METHOXYL		RATIO, METHOXYL TO GALACTURONIC ACID IN A.I.S.	RATIO, METHOXYL ⇆ CARBOXYL GROUPS TO GALACTURONIC ACID IN A.I.S.	ESTERIFIED POLYGALACTURONIC ACID (% A.I.S.)
			% A.I.S.	Equivalent to total carboxyl groups (% A.I.S.)			
	Valencia orange vesicles						
1	6.08	26.82	2.79	4.21	0.104	0.157	66.27
2	6.15	27.13	2.77	4.30	.102	.158	64.42
3	5.82	25.67	2.94	4.35	.115	.169	67.59
Mean	6.02	26.54	2.83	4.29	0.107	0.161	66.09
	Navel orange vesicles						
1	5.50	24.26	3.10	3.90	0.128	0.161	79.49
2	5.38	23.73	3.15	3.92	.133	.165	80.36
3	5.61	24.75	3.05	3.87	.123	.156	78.81
Mean	5.50	24.25	3.10	3.90	0.128	0.161	79.55
	Navel orange pulp						
1	6.54	28.85	3.97	4.85	0.138	0.168	81.86
2	6.89	30.39	3.97	4.97	.131	.164	79.88
3	6.74	29.73	3.92	4.92	.132	.165	79.67
Mean	6.72	29.66	3.95	4.91	0.134	0.166	80.47
	Lemon pulp						
1	8.40	37.05	4.71	6.09	0.127	0.164	77.34
2	8.55	37.71	4.65	6.04	.123	.160	76.99
3	8.45	37.27	4.76	6.13	.128	.164	77.65
Mean	8.47	37.34	4.71	6.09	0.126	0.163	77.33

the alcohol-insoluble solids, as they are extracted from the vesicles and pulp with 80% alcohol.

The data of table 3 show the relation of the amount of uronic acid anhydride to the amount of furfural in the alcohol-insoluble solids. Uronic acid anhydride determinations were calculated from both the CO_2 and the saponification values of the alcohol-insoluble solids. The values calculated from the percentages of CO_2 are much higher than those calculated from the saponification values. The lower values can be partly explained by the fact that some of the pectin is combined with inorganic cations, through the carboxyl groups; this signifies that not all the carboxyl groups are completely

TABLE 3

RELATION OF URONIC ACID ANHYDRIDE TO FURFURAL CONTENT OF ALCOHOL-INSOLUBLE SOLIDS (A.I.S.) OF CITRUS VESICLES AND PULP

Sample no.	Total CO_2 (% A.I.S.)	Uronic acid (galacturonic) anhydrides		Furfural			Pentosans, equivalent to furfural from substances other than uronic acid anhydrides ×1.72 (% A.I.S.)
		Equivalent to $CO_2 \times 4.0$ (% A.I.S.)	Equivalent to methoxyl ×5.677 (% A.I.S.)	Equivalent to uronic acid anhydrides ×0.235 (% A.I.S.)	Total determined (% A.I.S.)	From substances other than uronic acid anhydrides (% A.I.S.)	
	Valencia orange vesicles						
1	6.08	24.32	15.84	5.72	11.70	5.98	10.28
2	6.15	24.60	15.73	5.78	12.15	6.37	10.96
3	5.82	23.28	16.69	5.47	11.58	6.11	10.51
Mean	6.02	24.07	16.09	5.66	11.81	6.15	10.58
	Navel orange vesicles						
1	5.50	22.00	17.60	5.17	12.44	7.27	12.50
2	5.38	21.52	17.88	5.06	12.65	7.59	13.05
3	5.61	22.44	17.31	5.27	12.72	7.45	12.81
Mean	5.50	21.99	17.60	5.17	12.60	7.43	12.78
	Navel orange pulp						
1	6.54	26.16	22.53	6.15	14.58	8.43	14.50
2	6.89	27.56	22.53	6.48	13.84	7.36	12.66
3	6.74	26.96	22.25	6.34	14.30	7.96	13.69
Mean	6.72	26.89	22.44	6.32	14.24	7.92	13.62
	Lemon pulp						
1	8.40	33.60	26.74	7.90	13.86	5.96	10.25
2	8.55	34.20	26.40	8.04	14.53	6.49	11.16
3	8.45	33.80	27.02	7.94	14.05	6.11	10.51
Mean	8.47	33.87	26.72	7.96	14.15	6.19	10.64

methoxylated. The values for the uronic acid anhydride were obtained by multiplying the percentages of CO_2 in the alcohol-insoluble solids by the factor 4.0. The percentages of total furfural equivalent to the uronic acid anhydride vary considerably in the different samples. The percentages of furfural derived from substances other than uronic acid, when multiplied by the factor 1.72, give the pentosan values of the alcohol-insoluble solids. The pentosan values may be expressed as pentoses if the furfural values are multiplied by the factor 1.95 instead of 1.72. The total furfural yielded by the alcohol-insoluble fractions of the vesicles and pulp ranged from a mean of 11.81% for Valencia orange vesicles to a mean of 14.24% for navel orange pulp. The mean values for Valencia and navel orange vesicles agree satisfactorily with the data previously reported by SINCLAIR and BARTHOLOMEW (12).

PECTIN CONTENT.—The water-soluble and acid-soluble pectins of the alcohol-insoluble fractions of citrus vesicles and pulp are reported as calcium pectate (table 4). Although hot water did not extract as much pectin as did dilute hydrochloric acid solution, the calcium pectate obtained from the aqueous extract was a better product and more easily purified than that from the acid extract. With few exceptions, primarily in lemon pulp, the calcium pectates from the aqueous extracts contained higher percentages of CO_2, and of calcium and furfural, than those from the acid extracts. The pulp contained more pectin than the vesicles, owing to the fact that the pulp samples included the central axis, which contains an albedo-like substance high in pectin.

The degree of purity of the calcium pectates can also be observed from the ratios of calcium to CO_2. The theoretical ratio of calcium to CO_2 is 0.455. The ratios from the water-soluble pectin show satisfactory agreement with the theoretical value; except for lemon pulp, the ratios from the acid-soluble pectin tend to be lower than the theoretical value.

The composition of the calcium pectates can be ascertained from their respective CO_2, calcium, and furfural values. Yields of CO_2 from the calcium pectates of the aqueous extracts are slightly higher than the 17.40% (mean) obtained by JOSEPH and BRYANT (6) from calcium pectates from lemon pectin. The calcium contents are higher than those usually reported for calcium pectate from citrus (5) and apple (3) pectins, and they are higher than would be expected from the CO_2 content—that is, on the basis that calcium pectate should contain 7.45% calcium. GADDUM (5) showed that the calcium content of calcium pectates prepared from pectins which had been repeatedly precipitated with alcohol approached a constant value of 7.5%. In his water-soluble albedo pectins, the initial high calcium values were associated with the presence of high ash values. The furfural content of the various calcium pectates is in accord with values reported by other investigators (10) on pectins from other sources, and they especially agree with the results of BRYANT *et al.* (2) on lemon pectin (*National Formulary*, ed. VIII), which contains a high content of galacturonic acid (87.6%). No claim is made that pectic acid can be freed of arabinose and galactose by precipitation as the insoluble calcium pectate. The complete removal of galactose and arabinose from pectin by hydrolysis (as by HCl in methyl alcohol) results in a degraded form of pectic acid. On the basis of the present analyses, the calcium pectate values given in table 4 represent, to a considerable degree of accuracy, the pectin content of these extracts.

TABLE 4

WATER-SOLUBLE AND ACID-SOLUBLE PECTIN OF ALCOHOL-INSOLUBLE SOLIDS (A.I.S.) OF CITRUS VESICLES AND PULP, DETERMINED AS CALCIUM PECTATE

Sample no.	Total CO_2 (% A.I.S.)	Water-soluble pectin as calcium pectate					Acid-soluble pectin as calcium pectate					Total calcium pectate determined (% A.I.S.)	CO_2 in calcium pectate (% A.I.S.)	Calcium pectate calculated from CO_2, %	CO_2 in residue (% A.I.S.)	Sum of CO_2 in calcium pectate and residue (% A.I.S.)	Undetermined CO_2 (% A.I.S.)
		Total (% A.I.S.)	Constituents, %			Ratio, Ca/CO_2	Total (% A.I.S.)	Constituents, %			Ratio, Ca/CO_2						
			CO_2	Calcium	Furfural			CO_2	Calcium	Furfural							
	Valencia orange vesicles																
1	6.08	9.60	18.40	8.47	20.71	0.460	17.88	17.13	7.43	17.99	0.434	27.48	4.83	79.4	0.63	5.46	0.62
2	6.15	9.76	18.21	8.25	20.71	.453	18.06	17.30	7.55	18.12	.436	27.82	4.90	79.7	.63	5.53	.62
3	5.82	9.80	17.82	8.27	21.63	.464	14.44	18.03	8.42	18.57	.448	24.24	4.35	74.7	.73	5.08	.74
Mean	6.02	9.72	18.14	8.33	21.02	0.459	16.79	17.49	7.80	18.23	0.439	26.51	4.69	77.9	0.66	5.35	0.66
	Navel orange vesicles																
1	5.50	10.58	17.22	7.71	21.08	0.448	16.52	16.00	6.86	18.60	0.428	27.10	4.46	81.1	0.38	4.84	0.66
2	5.38	11.41	17.86	7.72	21.44	.430	15.60	16.65	7.29	18.38	.438	27.01	4.64	86.2	.39	5.03	.35
3	5.61	10.71	17.64	7.78	21.86	.441	14.09	16.90	6.96	17.90	.412	24.80	4.27	76.1	.35	4.62	.99
Mean	5.50	10.90	17.57	7.74	21.46	0.440	15.40	16.52	7.04	18.29	0.426	26.30	4.46	81.1	0.37	4.83	0.67
	Navel orange pulp																
1	6.54	12.89	18.14	8.40	21.60	0.425	19.25	16.36	7.06	20.45	0.431	32.14	5.49	84.0	0.51	6.00	0.54
2	6.89	13.43	17.54	8.46	22.00	.443	20.94	16.50	7.13	17.88	.426	34.37	5.80	84.2	.44	6.24	.65
3	6.74	13.22	18.02	8.21	21.45	.453	20.72	16.74	7.08	18.73	.423	33.94	5.85	86.8	.41	6.26	.48
Mean	6.72	13.18	17.90	8.36	21.68	0.440	20.30	16.53	7.09	19.02	0.427	33.48	5.71	85.0	0.45	6.17	0.56
	Lemon pulp																
1	8.40	16.92	17.98	8.67	20.90	0.482	20.58	19.02	8.98	21.17	0.472	37.50	6.95	82.7	0.45	7.40	1.00
2	8.55	17.04	18.35	8.79	21.10	.479	18.33	19.30	8.61	21.46	.446	35.37	6.66	77.9	.64	7.30	1.25
3	8.45	16.25	18.19	8.69	20.88	.478	20.28	19.00	8.75	20.62	.460	36.53	6.81	80.6	.51	7.32	1.13
Mean	8.47	16.74	18.17	8.72	20.96	0.480	19.73	19.11	8.78	21.08	0.459	36.47	6.81	80.4	0.53	7.34	1.13

Total calcium pectate was determined as the sum of that determined in the aqueous and acid extracts (table 4). As might be expected, the Valencia and navel orange vesicles yielded lower amounts of calcium pectate than either the navel orange or the lemon pulps.

The amount of CO_2 equivalent to the total calcium pectate (table 4) ranged from a mean of 4.46% in the navel orange vesicles to a mean of 6.81% in the lemon pulp. It can be seen readily that the CO_2 of the calcium pectate did not account for the total CO_2 of the alcohol-insoluble fraction. The percentage of the total CO_2 determined as calcium pectate ranged from a mean of 77.9% in the Valencia orange vesicles to a mean of

TABLE 5

BALANCE SHEET SHOWING RELATION OF CARBON DIOXIDE IN ALCOHOL-INSOLUBLE SOLIDS (A.I.S.) TO AMOUNT DETERMINED IN CALCIUM PECTATE FROM AQUEOUS AND ACID EXTRACTS

Sample no.	Total CO_2 (% A.I.S.)	CO_2 in aqueous extract			CO_2 in acid extract			CO_2 in residue (% A.I.S.)	Sum of CO_2 fractions determined (% A.I.S.)	Percentage of total CO_2 determined
		Total (% A.I.S.)	Equivalent to Ca pectate in extract (% A.I.S.)	Percentage accounted for	Total (% A.I.S.)	Equivalent to Ca pectate in extract (% A.I.S.)	Percentage accounted for			
					Valencia orange vesicles					
1	6.08	2.00	1.77	88.5	3.53	3.06	86.7	0.63	6.16	101.3
2	6.15	2.04	1.78	87.2	3.53	3.12	88.4	.63	6.20	100.8
3	5.82	2.20	1.75	79.5	2.87	2.60	90.5	.73	5.80	99.6
Mean	6.02	2.08	1.77	85.1	3.31	2.93	88.5	0.66	6.05	100.5
					Navel orange vesicles					
1	5.50	2.00	1.82	91.0	3.04	2.64	86.8	0.38	5.42	98.5
2	5.38	2.08	2.04	98.0	3.12	2.60	83.3	.39	5.59	103.9
3	5.61	2.06	1.89	91.7	3.22	2.38	73.9	.35	5.63	100.3
Mean	5.50	2.05	1.92	93.6	3.13	2.54	81.3	0.37	5.55	100.9
					Navel orange pulp					
1	6.54	2.39	2.34	97.9	3.18	3.15	99.0	0.51	6.08	92.9
2	6.89	2.68	2.35	87.7	3.50	3.45	98.5	.44	6.62	96.1
3	6.74	2.64	2.38	90.1	3.51	3.47	98.9	.41	6.56	97.3
Mean	6.72	2.57	2.36	91.9	3.40	3.36	98.8	0.45	6.42	95.4
					Lemon pulp					
1	8.40	3.30	3.04	92.1	4.32	3.91	90.5	0.45	8.07	96.1
2	8.55	3.39	3.13	92.3	4.14	3.53	85.3	.64	8.17	95.5
3	8.45	3.36	2.96	88.1	4.18	3.85	92.1	.51	8.05	95.2
Mean	8.47	3.35	3.04	90.8	4.21	3.76	89.3	0.53	8.09	95.6

85.0% in navel orange pulp. The sum of the CO_2 in the total calcium pectate and in the residue is equal to the total amount determined. The undetermined CO_2, or the amount lost in chemical procedures, was obtained by subtracting these summation values from those for total CO_2. The loss that occurred ranged from 0.56 to 1.13% (mean values) of the alcohol-insoluble solids. On purification, the pectins (not calcium pectate) are repeatedly dissolved in water and subsequently precipitated by adding alcohol or acetone. Such a procedure does not always remove the extraneous material without the loss of pectin. This loss is especially significant when small amounts of pectin are involved and quantitative recovery is desired.

That some pectin is bound firmly to the cellulose-hemicellulose fraction is borne out by the amount of CO_2 in the residue. On an alcohol-insoluble-solids basis (table 4), the CO_2 remaining in the residue was lowest (mean, 0.37%) in navel orange vesicles and highest (mean, 0.66%) in Valencia orange vesicles. As shown in studies with lemon peel (**14**), this residual pectin can be separated from the residue by hydrolysis with HCl. In addition to the pectin, the HCl hydrolyzes considerable hemicellulosic material that is hard to free from the pectin when the latter is determined as calcium pectate.

CARBON DIOXIDE IN AQUEOUS AND ACID EXTRACTS OF ALCOHOL-INSOLUBLE SOLIDS.—The data in table 5 show the degree of efficiency with which the calcium ion precipitates pectin from the aqueous and acid extracts of the various alcohol-insoluble fractions. The mean amounts of CO_2 recovered as calcium pectate from aqueous extracts of Valencia and navel orange vesicles were 85.1 and 93.6%, respectively. The mean amounts of CO_2 recovered as calcium pectate from the acid extracts of navel orange vesicles and pulp were 81.3 and 98.8%, respectively.

The preceding results show that the uronic acid–CO_2 extracted from the alcohol-insoluble solids with water or dilute acid solution and precipitated as calcium pectate ranged between means of 81.3 and 98.8%. Undoubtedly, a portion of the loss in CO_2 occurred in the large volume of wash water used to free the calcium pectate of chlorides. The fact that most of the CO_2 occurred in the galacturonic acid of the calcium pectate indicates that a relatively small amount of uronide could be combined with the hemicellulose dissolved by the aqueous and dilute HCl solutions in the extraction process.

Another point worthy of note is the relation of the CO_2 of the alcohol-insoluble solids to the sum of the CO_2 of the various fractions. As shown in table 5, the sum of the total CO_2 in the aqueous extract, acid extract, and residue of the four groups of samples ranged between 95.4 and 100.9% (mean values) of the total CO_2 content of the alcohol-insoluble solids. Approximately 5% of the total CO_2 was lost in the extraction process.

The material that remains after extraction of the pectins from the alcohol-insoluble solids is composed chiefly of cellulose and hemicellulose, which, in turn, contain a comparatively small amount of firmly bound pectin that is difficult to extract and determine quantitatively. The extractants used to dissolve the pectin also dissolve various portions of the hemicellulose. This condition makes it difficult to extract and precipitate all the uronic acid–CO_2 as calcium pectate. The quantity of CO_2 in the residues of the various samples ranged between 0.37 and 0.66% (mean values) of the total alcohol-insoluble solids (table 5).

Discussion

The alcohol-insoluble solids of citrus vesicles and pulp yield only a slight test for starch. In the absence of an appreciable quantity of starch, procedures used for the isolation of the various fractions in the alcohol-insoluble solids are considerably simplified and shortened. The chief difficulty in these procedures is the isolation of the pectin from the cellulose-hemicellulose complex. As the molecular composition of pectin varies from one sample of pectin to another, the uronide represented by the last traces of CO_2 of the alcohol-insoluble solids yields a calcium pectate of a variable composition. At the present time there are no practical chemical procedures for separating the various polygalacturonic acid components from extractives of pectin material.

The degree of esterification, or the methoxyl content, of the alcohol-insoluble solids is important in that the pectin content of the fraction can be determined. In his studies GADDUM (5) reported the degree of methylation of the extracted pectins from citrus albedo, pulp, and juice as the ratio of methoxyl to pectic acid. As pointed out by NORMAN (9), this method of expressing results is more desirable than that based upon the total weight of the sample. In the experiments reported in this paper the degree of methylation of the alcohol-insoluble solids is reported as the ratio of methoxyl groups to the galacturonic acid content. Since the galacturonic acid content is calculated from the uronic acid–CO_2 of the sample, and the methoxyl is determined by saponification, the ratio thus obtained is a fair estimate of the methoxyl content of the pectin in the alcohol-insoluble solids.

In a polygalacturonic acid molecule the number of carboxyl groups that are free, esterified, or combined with cations is not constant. This is confirmed by the results of GADDUM (5), who noted that at any stage of maturity the pectins of the albedo are more highly methylated than those of the juice. In the juice of the orange the maximum ratio of methoxyl to pectic acid was 0.120 in the pre-ripe stage, decreasing to a value of 0.097 in the fully mature stage. In the present investigation the ratios of methoxyl to polygalacturonic acid of the alcohol-insoluble solids of the vesicles and pulp are derived from the insoluble pectins of the vesicles and pulp, as well as from the soluble pectins of the juice.

Summary

1. The alcohol-insoluble fraction of the juice vesicles of Valencia and navel oranges, and of the pulp of navel oranges and lemons, has been isolated from the fresh material by extraction with 80% ethyl alcohol. This fraction, which contained the cellulose, most of the hemicellulose, and pectin, was lowest in Valencia orange vesicles (mean, 9.87%) and highest in navel orange pulp (mean, 15.56%), on a dry-weight basis.

2. Since the alcohol-insoluble fraction contains the pectin, most of the carbon dioxide which can be liberated by hydrolysis with 12% HCl, and all the methoxyl groups, occur in this fraction. The ratio of methoxyl to CO_2 was much lower in Valencia orange vesicles than in navel orange vesicles, navel orange pulp, or lemon pulp.

3. The carbon dioxide equivalent to the sum of the esterified and non-esterified carboxyl groups was equal to the total carbon dioxide of the alcohol-insoluble fraction obtained on hydrolysis with 12% hydrochloric acid.

4. The sum of the water-soluble and acid-soluble pectin (as calcium pectate)

of the alcohol-insoluble fraction of the vesicles and pulp samples ranged between means of 26.30% in navel orange vesicles and 36.47% in lemon pulp. These values are much lower than those for the total pectin calculated from the carbon dioxide and methoxyl values.

5. To establish criteria of purity, the calcium pectate values are accompanied by corresponding values for carbon dioxide, calcium, and furfural. The percentages of calcium in the calcium pectates are generally somewhat higher than the 7.50% usually reported for pure calcium pectate, and the percentages of carbon dioxide are generally slightly higher than the 17.40% reported for the pure compound. The furfural values are in accord with those reported by other investigators. The ratios of calcium to carbon dioxide of the calcium pectates from the water-soluble pectin show satisfactory agreement with the theoretical value; except in lemon pulp, the ratios of these substances from the acid-soluble pectin tend to be lower than the theoretical value.

6. The residue that remains after extracting the pectin from the alcohol-insoluble solids of the vesicles and pulp is composed of cellulose and hemicellulose and a comparatively small amount of firmly bound pectin that is difficult to extract and determine quantitatively.

7. The sum of the carbon dioxide in the aqueous extract, acid extract, and residue amounted to 95.4% and 100.9% (mean values) of the total carbon dioxide of the alcohol-insoluble solids in the vesicles and pulp, respectively. In the aqueous and acid extracts, calcium pectate accounted for more than 85% of the carbon dioxide dissolved by the extractants.

8. As a measure of the degree of esterification of the alcohol-insoluble fractions, the ratio of methoxyl to galacturonic acid was lowest in the Valencia orange vesicles (mean, 0.107) and highest (mean, 0.134) in navel orange pulp. In a polygalacturonic acid, the number of carboxyl groups that are free, esterified, or combined with cations is not a constant amount but varies considerably with the method of preparation.

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EFFECTS OF 2,4-D ON DRY WEIGHT, REDUCING SUGARS, TOTAL SUGARS, POLYSACCHARIDES, NITROGEN, AND ALLYL SULFIDE IN WILD GARLIC[1]

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Introduction

2,4-Dichlorophenoxyacetic acid (2,4-D) is the most versatile and the most widely used of the chemical herbicides. Though it has gained widespread acceptance and usage, its mode of action is as yet only partially understood. The basic effects which account for the herbicidal properties of "growth-regulating" compounds remain obscure, though much has been learned concerning their effects on general growth responses, respiration, dry weight, and changes in the various chemical components, particularly the carbohydrates. This study using a monocotyledon, wild garlic (*Allium vineale* L.), was planned to further understanding of the effects of 2,4-D on the physiology and chemical components of plants. Similar studies have been conducted principally on dicotyledons.

GROWTH RESPONSES.—BROWN (**3**), who studied the bean plant, and MITCHELL and BROWN (**13**), who took growth measurements of annual morning-glory, reported that the growth of leaves, stems, and buds was inhibited in both plants by treatment with 2,4-D. EAMES (**4**) reported that nutsedge (*Cyperus rotundus*) exhibited two types of response following treatment with 2,4-D: (*a*) some tissues and parts of organs, such as leaves, were stimulated to mature at once in abnormal form and (*b*) other tissues became abnormally meristematic and formed new abnormal tissue continuously for a considerable time. This type of response was shown particularly in the stems.

EFFECT ON RESPIRATION.—Though respiration was not studied directly in this experiment, an understanding of

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how this process is affected by 2,4-D is necessary to interpret food usage or storage. 2,4-D may stimulate or retard the respiration rate in plants. Concentration of 2,4-D in relation to plant susceptibility appears to govern the critical threshold between stimulation and inhibition. Under differing conditions of treatment with 2,4-D, both increased rates of respiration (**3, 7, 9, 21**) and decreased rates of respiration (**8, 11, 14, 23**) have been reported.

DRY WEIGHT.—By means not yet understood, 2,4-D and related growth-regulating substances upset the balance between synthesis and consumption of various plant foods as shown directly by their effect on dry weight. A decrease in dry weight of bean plants resulted after treatment with indoleacetic acid (**1**), and lowered dry weights were reported in beans (**3**), annual morning-glory (**13**), and soybean plants (**25**) following treatment with 2,4-D.

CHEMICAL COMPONENTS.—This loss in dry weight has been most clearly traced to the carbohydrates. There is a steady decline in starch and starchlike substances following herbicidal applications of 2,4-D (**13, 16, 18, 21, 26**) and usually an increase in the percentage of sugars immediately following treatment with 2,4-D (**16, 20, 26**). This initial increase is followed by a rapid and steady decline in the sugars (**20, 26**). TUKEY *et al.* (**24**) reported disappearance of starch from tissues of both bindweed and sow thistle. It was postulated that this decrease in starch might result from stimulation of hydrolysis by 2,4-D.

The role of proteins has not been so well established. It has been found that, with higher rates of 2,4-D application, the percentage of protein in the wheat grain was increased accordingly (**5**). Rates of applications of 2,4-D up to 4.6 pounds per acre were used. Other studies (**10, 19**) indicate that increase in protein content in the wheat grain is inversely related to reduced yield of the crop as a result of herbicidal injury. When calculated on total nitrogen content of the grain, the number of pounds of protein synthesized in no case increased, and in several cases total protein per acre, as determined by grain yield, was significantly reduced by 2,4-D treatments.

Wild garlic is undesirable in pastures and small grains primarily because of objectionable odors and flavors it causes in the milk and grain products. According to STOLL and SEEBECK (**22**), "alliin" is the substance in garlics which, upon decomposition by a specific enzyme, yields allicin, and this, in turn, the highly volatile and odorous allyl sulfides. Alliin has an empirical formula of $C_{12}H_{23}O_7N_2S_2$. WYLIE and HARRISON (**27**) identified allyl sulfide as the substance responsible for the onion flavor in milk, and HAGGARD and GREENBERG (**6**) indicated that the odor from cultivated garlic is caused by diallyl sulfide.

Material and methods

TREATMENT

The area sampled was in the North Carolina State College Dairy Farm pasture near Raleigh and had a dense stand of wild garlic. In the treatment on April 7, 1949, 3 pounds of 2,4-D (acid equivalent) were applied per acre using water as a carrier. The triethanolamine formulation of 2,4-D was used.[4] The solution was applied by a boom equipped with Monarch no. 22 nozzles, delivering 11 gallons per acre. The area was sprayed twice, being crossed in opposite directions to insure uniform coverage. Double

[4] Supplied by the du Pont Semesan Laboratory.

spraying gave a total application equivalent to 22 gallons per acre.

Sampling

LENGTH-OF-PLANT MEASUREMENTS.—The longest six plants in one of the treated samples and also in one of the check samples were measured. Measurements were made from the bulb base to the green leaf tip. As garlic matures, the leaf tip dies and often breaks off. Therefore, the dead leaf part was not included.

BULB COUNTS.—Number of bulbs per plant was determined from the first six plants dug in the treated area and also in the check area. Bulb counts were continued a month after other sampling was completed.

SAMPLES FOR CHEMICAL ANALYSES.—New garlic plants were still emerging during the sampling period, so only the more mature plants were taken which provided reasonably uniform plants. Chronological sampling of treated and check plants was used to facilitate the study of changes in the plant that followed 2,4-D application. Samples were taken 6 hours, 1 and 4 days, and 1, 2, 3, 4, and 5 weeks after application.

Samples consisted of sixty plants each and were taken from both treated and nontreated areas. Samples were taken in duplicate for the first three periods and in triplicate thereafter. Duplicate samples were stored dry, and the third sample was stored in absolute alcohol for allyl sulfide determination.

Preparation and storage of samples

Soil temperature was taken at a 3-inch depth to approximate bulb depth. Roots and bulbs were washed thoroughly in water adjusted to that temperature, and cleaned plants were wrapped in wet sacks of the same temperature and promptly placed in the drying oven or in boiling alcohol.

SAMPLES STORED DRY.—Samples to be stored dry were placed in a preheated, forced-air circulating oven at 101° C. for 30 minutes. The oven was preheated sufficiently so that the temperature was approximately constant through this period. Then plants were removed, the bulbs split open longitudinally to facilitate drying, and returned to the dryer. Drying was continued for 20 hours at 70° C., and dry weights were recorded. The dry weight per plant was calculated by dividing the total dry weight of the sample by 60. The samples were stored whole in sealed glass containers.

SAMPLES STORED IN ALCOHOL.—Samples to be stored in alcohol were separated into tops and bulbs. Fresh weights were taken, and these samples were then placed in glass containers in which they were to be kept. Samples were treated with absolute alcohol in accordance with the method outlined in paragraph 12.1 (b), *Official Methods* (2).

Methods of chemical analyses

Bulbs and tops of dried samples were separated. Roots represented little weight but were placed with tops. Each separation was ground in a Wiley mill equipped with an 80-mesh screen. Samples were redried at 70° C. for 12 hours to insure uniform dryness.

The percentages of dry weight in bulbs and tops represented by the various components were determined by chemical analysis. Results from duplicate samples were totaled and averaged. The milligrams of the component in bulbs or tops were calculated by multiplying the percentage by their respective dry weights. The milligrams per total plant were calculated as the sum of milligrams in the tops and bulbs. The per-

centage of any component in the total plant was calculated by dividing the milligrams of the component per total plant by the milligrams of dry weight per plant.

An adaptation of the Soxhlet extraction method (12) was used to separate alcohol-soluble carbohydrates from alcohol-insoluble carbohydrates. Extraction was made for 8 hours with 80% ethyl alcohol.

The alcoholic extract used for sugar determinations was cleared with lead acetate .The precipitate was centrifuged down, sodium oxalate added in slight excess, the precipitate re-centrifuged, and the cleared liquid poured off and made up to volume.

REDUCING SUGARS.—The percentage of reducing sugars was determined by the method outlined by POE and EDISON (15), which permits colorimetric determination and was particularly useful in this study because of the time saved.

TOTAL SUGARS.—The percentage of total sugars was obtained from another aliquot of the cleared extract by enzymatic inversion at room temperature for 12 hours with Wallerstein Red Label invertase. Total reducing power was determined colorimetrically as above.

TOTAL POLYSACCHARIDES.—The percentage of total polysaccharides was determined from a sample of the dried, alcohol-insoluble residue which was hydrolyzed in $1\frac{1}{4}\%$ sulfuric acid by being placed in a boiling water bath for 2 hours. The reducing power was determined colorimetrically as described above. A factor of 0.9 was used to calculate the total polysaccharides.

TOTAL NITROGEN.—The percentage of total nitrogen was determined from the dried material. Analyses were made by the Kjeldahl method (2). The crude protein value was obtained by multiplying total nitrogen by the factor 6.25.

ALLYL SULFIDE AND RELATED SULFIDES.—No established method was available for determination of allyl sulfide. A turbidity method was used, as adapted from RIBAS *et al.* (17), and was reasonably satisfactory for this study. The method used was as follows: The alcohol in which the garlic plants had been stored for 4 months was separated from solid plant parts by filtering. Sufficient 80% ethyl alcohol was added to it so that 1 gm. of green plant material was represented by 5 cc. of the alcoholic extract. A 5-cc. aliquot of this extract was used in each determination. Two drops of saturated lead acetate were added to precipitate the proteins. Sufficient sodium oxalate was added to precipitate the remaining lead acetate. The sample was cooled in cold water for 3 minutes and then centrifuged for 3 minutes. The cleared extract was poured off, and 5 cc. of 80% alcohol and 10 cc. of gold chloride (1:1000) were added. After 10 minutes the turbidity was determined by measuring the percentage transmission in the colorimeter. The amount of allyl sulfide and closely related sulfides was determined from a graph previously prepared by measuring the turbidity caused by known amounts of allyl sulfide (C. P. Eimer and Amend). Determinations gave directly the parts per million allyl sulfide in the green material. By multiplying the parts per million sulfides in each gram by the total green weight, the milligrams of sulfides per plant were obtained.

Results and discussion

Data included dry weight, reducing sugars, total sugars, total polysaccharides, total nitrogen, and allyl sulfide and related sulfides. In addition, observational notes and data were taken on

appearance and length of plants and on number of bulbs formed per plant as influenced by treatment.

GENERAL APPEARANCE.—The first effect after spraying was apparent the second day. The outer covering of bulbs of treated plants felt slippery. This characteristic was not noted again until 3 weeks after application, when the sliminess became progressively more pronounced. In the third week the plants appeared to be recovering and new growth started. By the fourth week, however, the tops were becoming brittle and hard, and many were lying on the ground. By the fifth week most tops were still alive but prostrate and in a much weakened condition.

LENGTH OF PLANTS.—Compared with plants not treated, the rate of elongation was slowed by 2,4-D, indicating an inhibitory effect (table 1). Treated plants continued to elongate until the fourth week after application. By this time leaf tips had turned brown, and leaves were drying from the tips downward. Since measurements were made from the base of the bulb to the uppermost green tissue, the recorded reduction in length probably resulted chiefly from dying back of the leaf tip rather than a shortening of the plant.

Statistical analysis indicated a significant difference in length at the 1% level as early as 4 days after application (table 1). The difference in length between treated and check plants gradually increased through the rest of the experiment.

NUMBER OF BULBS PER PLANT.—It appears from the data (table 1) that treated plants continued to form new bulbs for several days and then ceased. The rapid formation of new bulbs immediately following application of the chemical could be associated with the maturation process. Variation in bulb counts in the treated area after the first 4 days was probably due to sampling error as suggested by the fact that counts in the treated area at 4 days and after 9 weeks gave the same average number of bulbs.

Untreated plants continued to form new bulbs and at the end of the experiment had more than double the number of bulbs found on treated plants.

TABLE 1

AVERAGE LENGTH (INCHES) AND AVERAGE NUMBER OF BULBS OF WILD GARLIC PLANTS[a] AFTER AN APPLICATION OF 2,4-D ON APRIL 7, 1949

TIME AFTER APPLICATION	LENGTHS OF PLANTS		NUMBER OF BULBS PER PLANT	
	2,4-D treated	Check	2,4-D treated	Check
6 hours	23.17	23.0	2.50	2.50
1 day	23.42	23.91	2.83	2.50
4 days	25.67**	30.67	3.17	3.00
1 week	27.25**	33.00	3.17	3.17
2 weeks	28.33**	33.50	3.00*	4.00
3 weeks	29.33**	34.67	3.50	4.00
4 weeks	30.67**	39.00	3.50*	4.50
5 weeks	27.17**	44.50	3.33*	4.50
7 weeks	.....	.....	3.33*	4.83
9 weeks	.....	.....	3.17**	6.83

[a] Each number the average of six plants.
* Significantly different from check at 5% level.
** Significantly different from check at 1% level.

This phase of the experiment was not planned to determine control measures. It is clear, however, that April 7 is too late for effective treatment of wild garlic in North Carolina. Even though treated at the heavy rate of 3 pounds of 2,4-D acid equivalent per acre, the plants each were able to produce an average of more than three bulbs. Formation of these bulbs was either initiated or complete prior to application of 2,4-D.

DRY WEIGHT.—Consecutive dry-weight measurements indicate the type and rate of metabolic processes within

the plant. After the first day dry weight of both tops and bulbs of 2,4-D–treated plants decreased (fig. 1), indicating predominance of the catabolic processes.

Losses in dry weight have been previously determined following treatment with 2,4-D. Since wild garlic is only moderately susceptible to 2,4-D, a heavy rate of application of 2,4-D was possible without rapidly killing the plants. Even though 3 pounds of 2,4-D (acid equivalent) per acre were applied, the plants were still alive after 5 weeks. This gave ample time for the chemical to exert its physiological effects prior to their death, and it was possible to make measurements that would have been less pronounced had the plants died in a few days.

REDUCING SUGARS, TOTAL SUGARS, AND POLYSACCHARIDES.—Reducing sugars probably supply the most ready source of energy for the plant. Therefore, any factor that speeds its catabolic processes may readily upset the equilibrium between usage and storage of sugars.

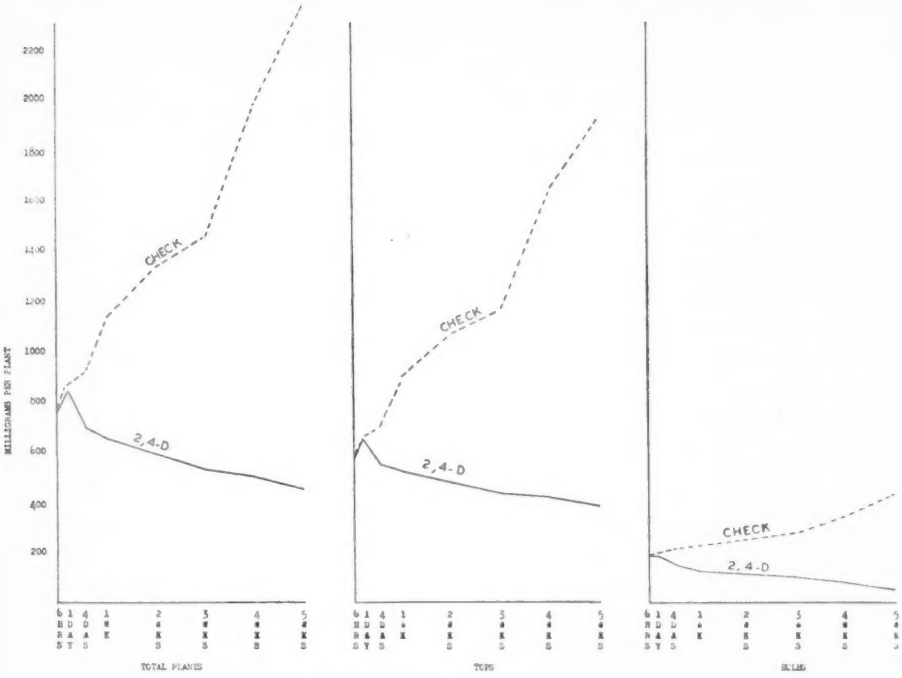


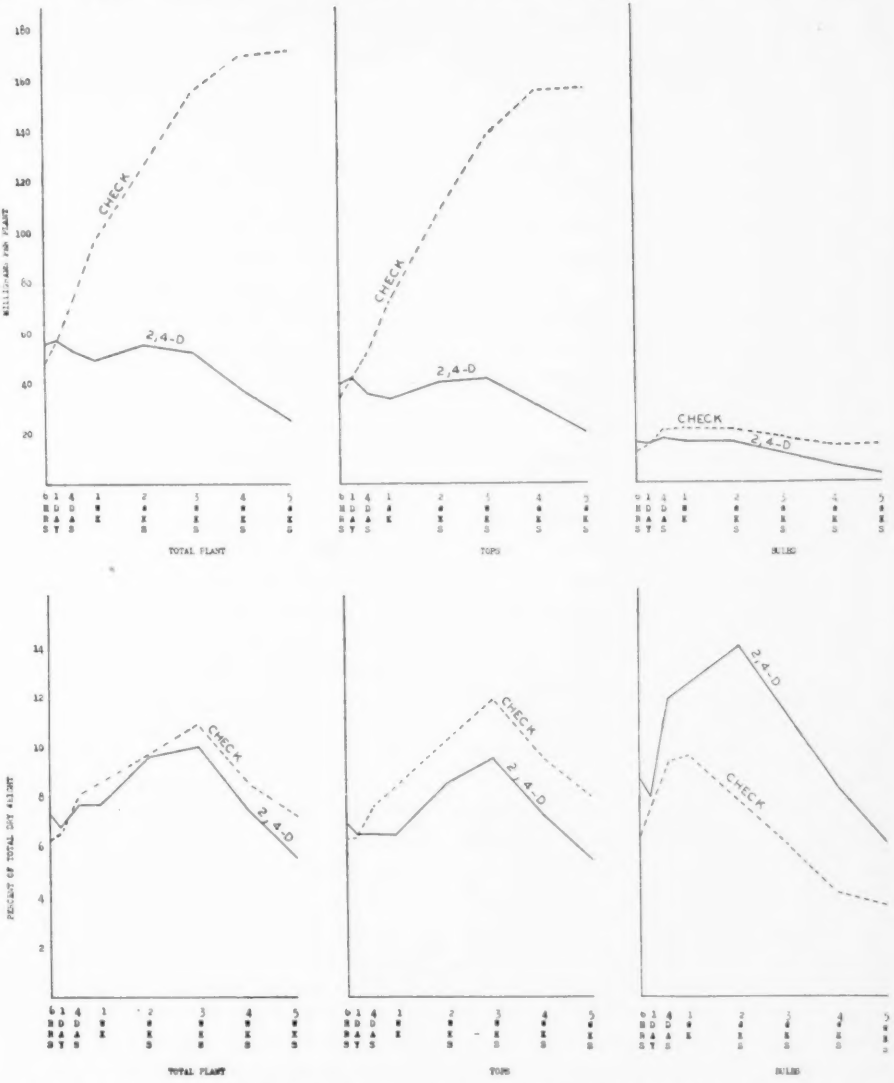
FIG. 1.—Dry weight per garlic plant treated with 2,4-D compared with check plants, at indicated times after application.

The milligrams of reducing sugar did not increase after the first day in treated plants (fig. 2); the general trend was downward when considered on a weight basis. In check plants the total weight of reducing sugars increased along with an increase in dry weight.

Figure 3 shows the percentage of the total dry weight which was made up of reducing sugars. For the plant as a whole there was but little difference in these percentages between treated and

check plants. The difference was somewhat more pronounced toward the end of the experiment, when 2,4-D–treated plants contained the lower percentage.

Tops of check plants stored a lower percentage of carbohydrates as polysaccharides than did bulbs. Thus, if the catabolic processes exceed the anabolic processes as a result of 2,4-D treatment, the readily available carbohydrates would be expected to be depleted more rapidly in tops than in bulbs (fig. 7).



FIGS. 2, 3.—Reducing sugars in garlic plants treated with 2,4-D compared with check plants, at indicated times after application. Fig. 2 (*above*), milligrams per plant; fig. 3 (*below*), percentage of total dry weight.

Also, 2,4-D may increase the rate of hydrolysis of the polysaccharides to the reducing sugars. A combination of these factors may account for the higher percentage of reducing sugars in treated bulbs and the lower percentage in treated tops.

Neither the percentage nor the total weight of reducing sugars continued to increase after the third week along with the increasing dry weight of the check plants. This would indicate that with usual maturity of the plant a larger proportion of the carbohydrates is stored as more "stable" carbohydrates. Such increases were found in the total polysaccharides.

There was a reduction in total sugars per plant up to 1 week after treatment with 2,4-D and a slight increase during the second week, followed by a downward trend thereafter (fig. 4).

The percentage of total sugars in the entire plant (fig. 5) decreased for 4 days after treatment. Then there was a gradual increase until the third week, after which the percentage again dropped. Except at the beginning, the percentage of total sugars in treated plants was at a lower level than in check plants. Samples taken in the third, fourth, and fifth weeks showed a slightly higher percentage in bulbs from treated plants than in those from check plants.

It should be emphasized that, even though treated plants did occasionally show a higher percentage of the various sugar fractions than did control plants, 2,4-D–treated plants contained a much lower quantity per plant when the calculation was on a weight basis.

In treated plants the milligrams of total polysaccharides per plant slightly increased and then were rapidly reduced during the first week (fig. 6). After the first week the reduction continued but at a less rapid rate. In the check plants the milligrams of total polysaccharides increased along with the increasing dry weight.

During the first 2 weeks of the experiment there was but little difference between treated and check plants in percentage of total polysaccharides (fig. 7). Analysis of samples taken 3, 4, and 5 weeks after treatment showed a gradual increase in this difference.

Bulbs from treated and check plants exhibited a considerable difference, particularly after the second week. The percentage of total polysaccharides rapidly increased in bulbs from check plants after 2 weeks, whereas bulbs from treated plants showed no such increase.

The reduction in amount of total polysaccharides in treated plants suggests increased usage by the plants through stimulated catabolic processes.

TOTAL NITROGEN.—There was but little difference between treated and check plants in the amount of total nitrogen present the first day after treatment (fig. 8). Both increased nearly the same amount. After the first day, however, treated plants rapidly and steadily lost in weight of the nitrogen present. The trend of the total nitrogen closely resembles that of dry weight. Since the nitrogen (as a component of proteins) and protoplasm of the plant are interdependent, this relationship is to be expected.

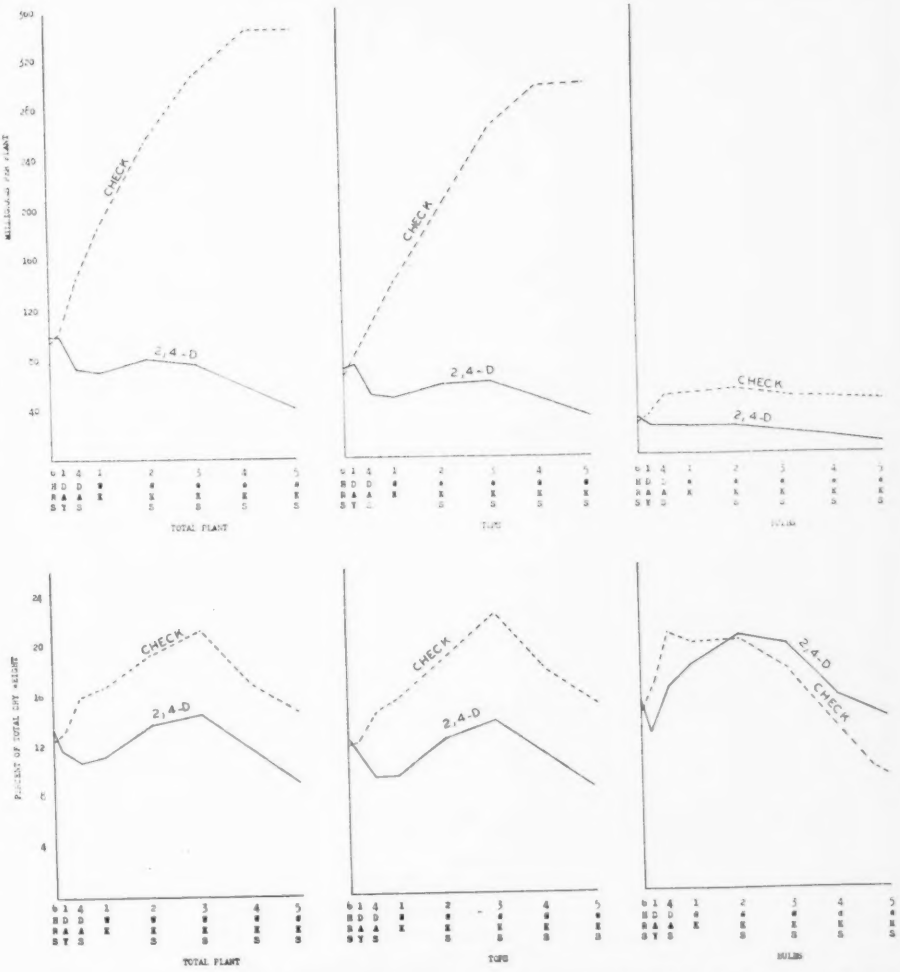
Tops exhibited trends similar to those of the entire plant. Bulbs from treated plants showed a slow but constant reduction in milligrams of nitrogen present. The data suggest that catabolic processes are such that even the nitrogenous substances of treated plants are broken down. The method by which nitrogen is lost needs further investigation.

In the 2,4-D–treated plants the per-

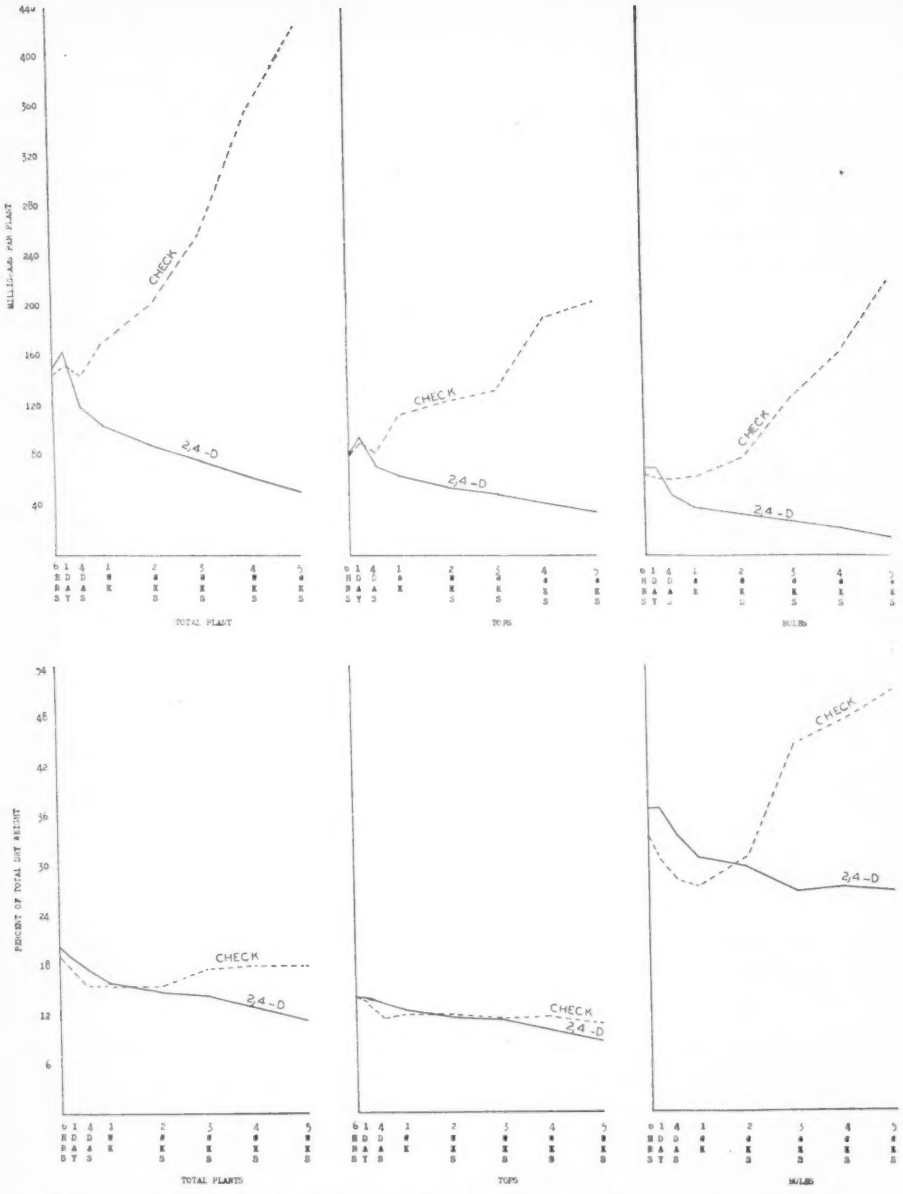
centage of total nitrogen gradually increased for the first week following treatment (fig. 9). This was followed by a steady reduction in the percentage of total nitrogen present.

Plant tops exhibited trends similar to those for the entire plant. The percentage of total nitrogen in bulbs from treated plants gradually increased, except for a small reduction 1 day after treatment. After 1 week the percentage of nitrogen in treated plants was consistently higher than that in check plants.

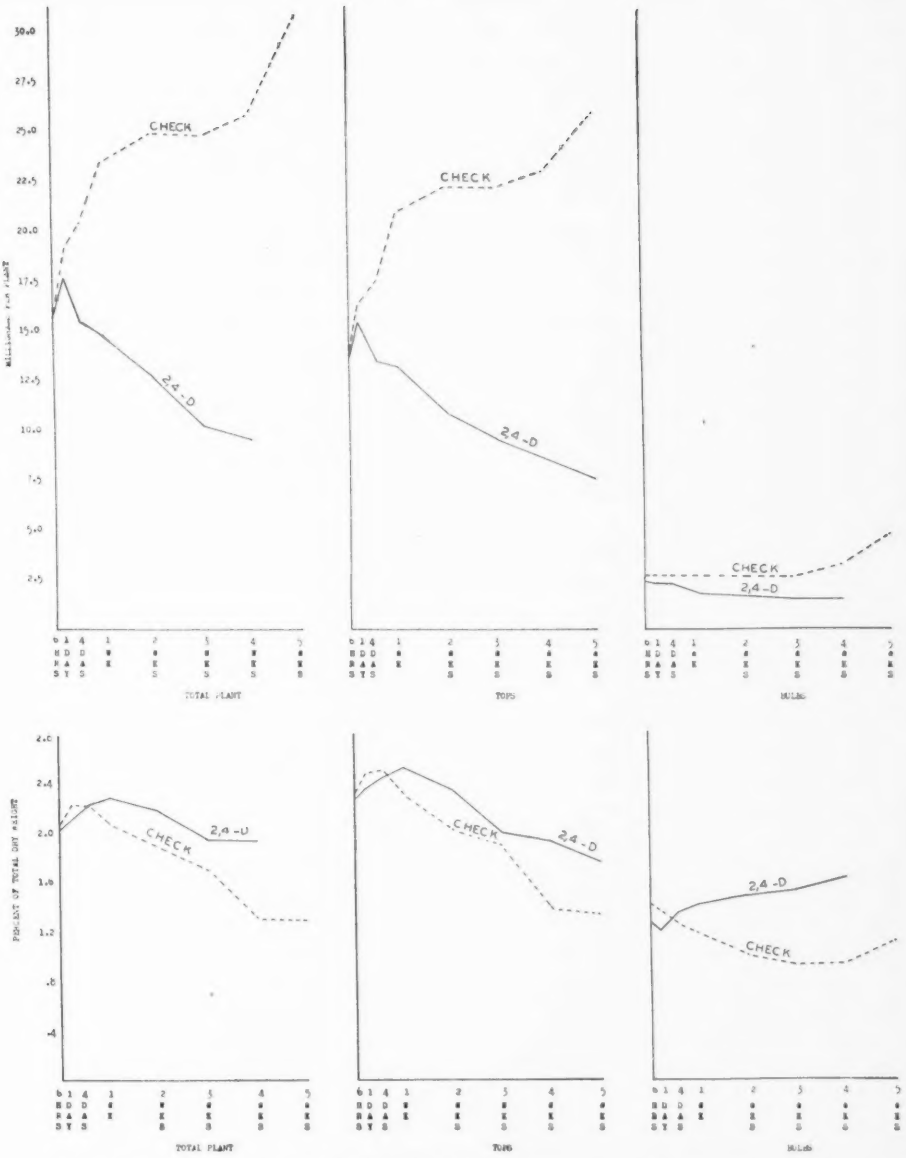
As discussed above, the 2,4-D treatment resulted in a loss of total milligrams of nitrogen per plant. Yet, 1 week after treatment, the percentage of nitrogen in treated plants was higher than in check



FIGS. 4, 5.—Total sugars in garlic plants treated with 2,4-D compared with check plants, at indicated times after application. Fig. 4 (*above*), milligrams per plant; fig. 5 (*below*), percentage of total dry weight.



FIGS. 6, 7.—Total polysaccharides in garlic plants treated with 2,4-D compared with check plants, at indicated times after application. Fig. 6 (*above*), milligrams per plant; fig. 7 (*below*), percentage of total dry weight.



FIGS. 8, 9.—Total nitrogen in garlic plants treated with 2,4-D compared with check plants, at indicated times after application. Fig. 8 (*above*), milligrams per plant; fig. 9 (*below*), percentage of total dry weight.

plants. Loss in weight of the carbohydrate fractions was relatively more rapid than for the nitrogen fraction, suggesting that enzyme systems regulating catabolism of both are affected by 2,4-D treatment and, further, that the carbohydrate fraction is then more rapidly utilized than the nitrogen fraction. This results in the relatively higher percentage of the nitrogen fraction. Since protoplasm is nitrogenous and usually considered rather stable in oxidative processes, this fraction would be expected to resist catabolism. This higher percentage of nitrogen in treated plants is not a result of an increased rate of protein synthesis, since milligrams of nitrogen per plant decreased following treatment with 2,4-D.

ALLYL SULFIDE AND SIMILAR SULFIDES.—Reports from farmers indicate that, after pastures have been treated with 2,4-D to control wild garlic, milk from cattle grazing in the pastures is often tainted little or not at all by the weed. This suggests that the herbicide directly destroys the sulfides responsible for the disagreeable flavor and smell or that the weed becomes unpalatable to the animals. Allyl sulfide, as previously shown, is thought to be the principal ingredient causing the odors and off-flavors. Related chemicals as well as other sulfides may be partly responsible, and the test used would also react with other organic sulfides present. Therefore the term "allyl sulfide and related sulfides" is used.

Data (fig. 10) show that the treated plants, as a whole, have less allyl sulfide than check plants. Between 4 and 5 weeks after treatment there was a definite reduction in the amount of the chemical present in treated plants. Data indicate that at least up to the fourth week there was a sufficient amount of the sulfides present to cause off-flavors in milk if the garlic plants were eaten by cattle.

Tops showed a trend similar to that of the entire plant. Bulbs from treated plants, however, showed a higher content of sulfides than did bulbs from check plants.

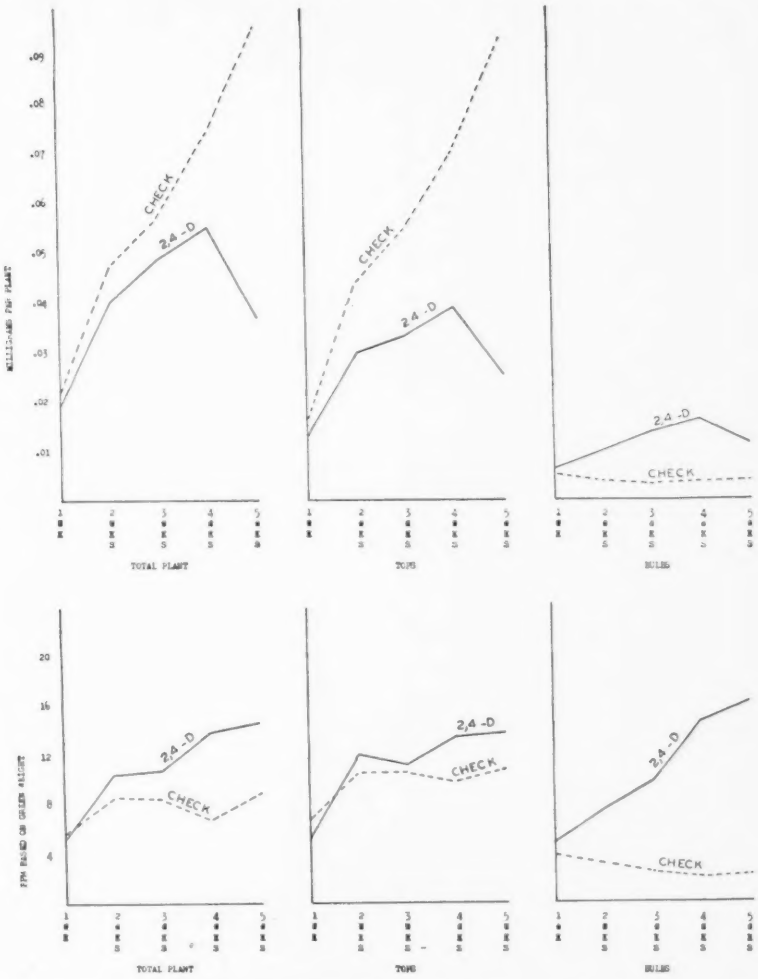
The parts per million of allyl sulfide in treated garlic plants rapidly increased following treatment with 2,4-D. This suggests that the oil-like sulfides resisted catabolism and accumulated as the plant lost weight; this resulted in an increase in their relative concentration (in terms of p.p.m.). Thus for equivalent green weights treated plants actually had a higher percentage of sulfides than did check plants.

QUANTITATIVE AND PERCENTAGE MEASURES OF THE VARIOUS COMPONENTS FOR THE WHOLE PLANT.—It is helpful to consider the various components together to give a better picture of the plant as a single complete organism. Therefore the total sugars, total polysaccharides, crude protein (nitrogen $\times$ 6.25), and dry weight are shown in the histogram in figure 12. These components all were gradually reduced after the first day following 2,4-D treatment, indicating that catabolic processes exceeded the anabolic processes for all four fractions in the treated plants.

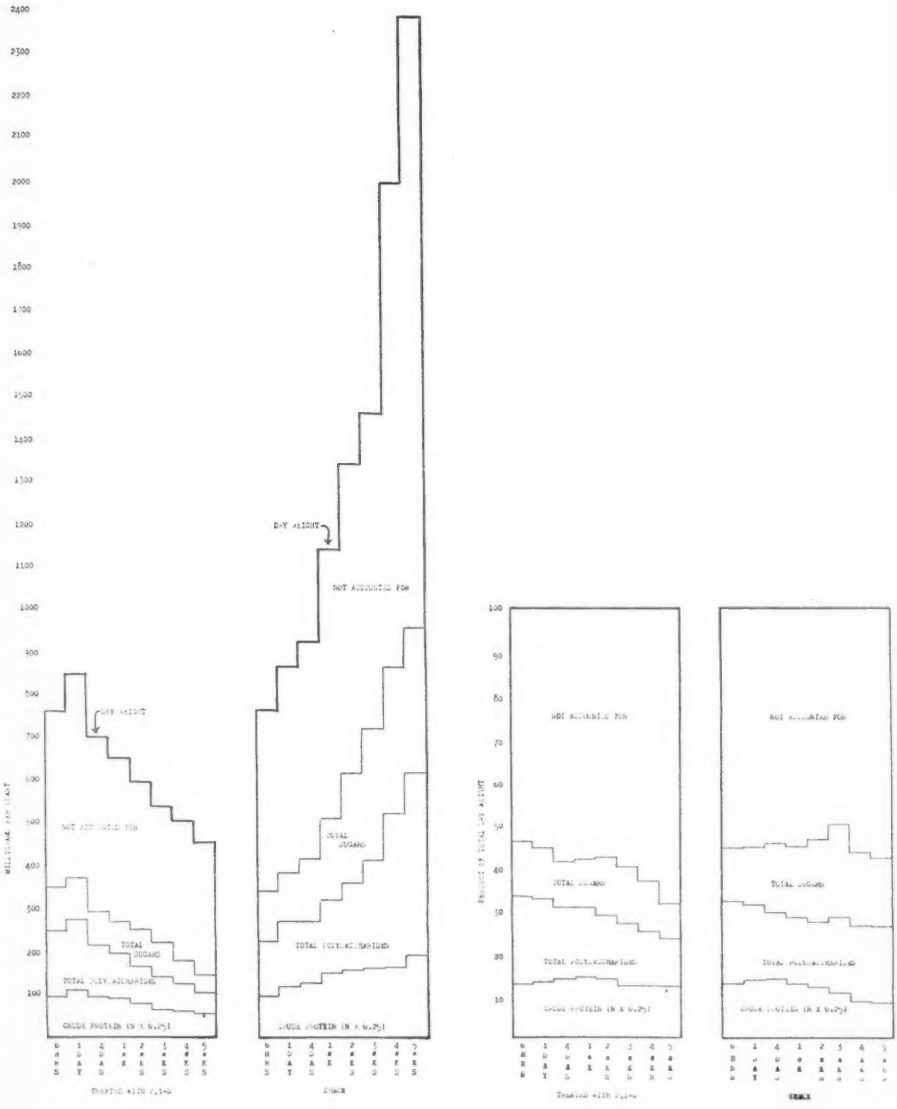
The histogram shows that in treated plants the total polysaccharides were reduced proportionately the most and the crude protein the least, total sugars being intermediate. If the metabolic processes influenced by 2,4-D are enzyme-controlled and result from increased activity of the oxidative enzyme systems, this can be explained. Protoplasm is nitrogenous and resists oxidation and is usually considered reasonably stable within the living plant. Much of the total polysac-

charides can be hydrolyzed to sugars, and an overly active enzyme system might conceivably convert polysaccharides to sugars. Therefore the quantity of sugars remains relatively high as long as there is an abundant source of polysaccharides. An increased rate of respiration must be assumed. Otherwise sugars would accumulate instead of being broken down to the usual oxidized products, CO_2 and H_2O.

Check plants showed an increase of each component, usually proportionately as the dry weights increased. Even in growth of check plants, total polysaccharides exhibited the greatest change,



FIGS. 10, 11.—Allyl sulfide and similar sulfides in garlic plants treated with 2,4-D compared with check plants, at indicated times after application. Fig. 10 (*above*), milligrams per plant; fig. 11 (*below*), parts per million based on green weight.



FIGS. 12, 13.—Dry weight, total sugars, total polysaccharides and crude protein (N × 6.25) in garlic plants treated with 2,4-D compared with check plants, at indicated times after application. Fig. 12 (*left*), milligrams per plant; fig. 13 (*right*), percentage of total dry weight. Asterisk = estimated, sample for fifth week ruined.

crude proteins the least, and total sugars were intermediate.

The percentages of the three foods discussed above are shown in figure 13. They varied much less in percentage than in total quantity (fig.12). Thus the delicate balance of the various components is reasonably well maintained within the plant as far as percentage of the various fractions is concerned, regardless of the fact that the metabolism of treated plants apparently was predominantly catabolic, whereas in check plants it was principally anabolic.

Summary

1. Wild garlic plants treated with 2,4-dichlorophenoxyacetic acid (2,4-D) were compared with plants not treated. 2,4-D slowed the rate of plant elongation and reduced bulb formation. The study indicated that earlier treatments would be essential in a control program. Dry weights were taken and chemical analyses were made of the tops and bulbs separately over a period of 5 weeks after application. On a weight-per-plant basis, treated plants lost in dry weight, total sugars, total polysaccharides, and total nitrogen. The quantity of allyl sulfide increased at nearly the same rate in treated and check plants until the fifth week, when it was reduced in treated plants.

2. Wild garlic plants not treated with 2,4-D increased in weight of the various components in much the same magnitude as the plant increased in total dry weight. In treated plants the "reserve" foods showed the greatest percentage of reduction. Thus total polysaccharides were reduced proportionately more than reducing sugars.

3. It is proposed that reserve carbohydrates were rapidly hydrolyzed to reducing sugars in treated plants. Reducing sugars were in turn oxidized through an active catabolic system, resulting in a total loss in dry weight. The trends suggest that with death of the plant the reserve carbohydrates and reducing sugars would be nearly exhausted.

4. The number of milligrams of total nitrogen per plant was reduced in treated plants. The method by which this loss took place needs experimental determination.

5. Percentage of total nitrogen was higher in treated plants than in check plants. This increase resulted from a more rapid utilization of the carbohydrate fraction than of the nitrogen fraction. Thus the higher percentage of nitrogen resulted from greater resistance of the nitrogen fraction than of the carbohydrate fraction to catabolism. The higher percentage of protein in treated plants did not result from increased rates of protein synthesis.

6. Allyl sulfide synthesis continued in treated plants for the first 4 weeks after treatment. The total quantity present was reduced in the fifth week. The quantity was ample to flavor milk products, if plants were eaten by dairy cows.

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NEW GROWTH-REGULATING COMPOUNDS
II. SUBSTITUTED BENZOIC ACIDS

C. E. MINARIK, D. READY, A. G. NORMAN, H. E. THOMPSON, AND J. FRED OWINGS, JR.

Introduction

The first comprehensive report of synthetic phenoxy and benzoic acid derivatives as plant growth-regulators was published by ZIMMERMAN and HITCHCOCK in 1942 (**8**). The phenoxy derivatives were found to be more active than the benzoic acid derivatives studied, and presumably for that reason the former group of compounds has received more attention by subsequent investigators. ZIMMERMAN and HITCHCOCK (**8**) reported that halogen substituents in the benzene ring of either benzoic or phenoxy acids increased the growth-regulating activity quite markedly. They further pointed out that in the case of phenoxy derivatives a halogen in the para position conferred greater activity on the molecule than the same substituent in the ortho position and that chlorine conferred greater activity than bromine in the same ring position.

Since the initial publication by ZIMMERMAN and HITCHCOCK there have appeared numerous papers dealing with benzoic acid derivatives and their effects upon plants. In 1946 the workers at Camp Detrick published a list of more than one thousand compounds that were investigated for growth-inhibitory activity (**6**). Many of these compounds were substituted benzoic acids. Since that time additional compounds have been tested by procedures which were designed to screen out compounds lacking inhibitory activity.

Material and methods

Most of the compounds tested were synthesized at Camp Detrick, although a few were obtained from commercial sources or from the Chemical-Biological Coordination Center. Since the aqueous solubility of this group is fairly low, conversion to the ammonium salt was resorted to when necessary to obtain the desired concentration.

The inhibitory effectiveness of approximately two hundred of these compounds was determined by at least two of the three following tests:

Test 1.—This is an adaptation of the READY and GRANT (**5**) cucumber bioassay. Concentration of test solutions employed ranged from 1 to 10 p.p.m.

Test 2.—This involves application of 5 mg. of test substance in aqueous solution to the soil in a 4-inch pot containing a single week-old Acorn squash plant. Limitation on weight of fresh tissue subsequently produced in comparison with an untreated control is the measure of effectiveness.

Test 3.—This involves spraying week-old Red Kidney bean plants with 2 ml. of an aqueous solution containing 50 μgm. of the compound under investigation. Criterion of effectiveness was the same as in test 2.

Although these tests provide quantitative data, the values that appear in tables 1 and 2 have been converted to descriptive terms because numerical values might convey an incorrect im-

pression of the relative effectiveness of certain compounds.

The compounds were separated into seven groups. Those having 76% or more of the inhibitory activity of 2,4-dichlorophenoxyacetic acid (2,4-D) in the same test are regarded as highly inhibitory (H), 51–75% as moderately inhibitory (M), 26–50% as slightly inhibitory (S), and 25% and below as inactive (I). Those compounds with negative values above −76% have been classified as highly stimulating (H.St.), from −51% to −75% as moderately stimulating (M.St.), and from −25% to −50% as slightly stimulating (S.St.).

TABLE 1

BENZOIC ACID DERIVATIVES WITH HIGH INHIBITORY ACTIVITY

NAME	ACTIVITY*		
	Test 1	Test 2	Test 3
2-bromo	H	.....	I
2-bromo-3-nitro	I	S	H
2-chloro-5-bromo	H	M	S
2-chloro-5-iodo	H	I	I
2-bromo-3-chloro-5-iodo	H	.....	M
2-bromo-3,5-dichloro	M	M	H
2-nitro-4-methyl-5-hydroxy	H	.....	I

* H = Highly active; M = Moderately active; S = Slightly active; I = Inactive.

Results and discussion

INHIBITION

As was to be expected, only relatively few of the compounds possessed sufficient activity to merit further interest. Approximately two hundred compounds were tested, but, of these, only seven can be classified as highly inhibitory and placed in the same category as 2,4-dichlorophenoxyacetic acid (2,4-D). Inasmuch as 2,4-D is the standard of comparison in these tests, the inhibition induced by these compounds was evaluated relative to inhibition by 2,4-D. The highly active compounds are listed in table 1, while a complete list of all compounds tested appears in table 2.

It is apparent from the tables that a compound may be active in one test and inactive in another. This is to be expected, since different plant species, rates and methods of application, and criteria of effectiveness are involved. The use of more than one test procedure increases the possibility of detecting active compounds and should be the practice in any screening program.

Although relatively few compounds were highly active, fully 50% of those tested displayed some degree of activity in at least one test.

Examination of the monohalogen substituted compounds discloses that, of the fourteen tested, only the 2-bromo compound was highly active, and this, moreover, only in the cucumber germination test.

Of the thirty-four monosubstituted nonhalogens tested, none was highly active, and only three were moderately active. These were the 2-methoxy, 2-phenylureido, and 4-ureido compounds.

Of the fifty-two disubstituted compounds containing at least one halogen atom, the 2-chloro-5-bromo, 2-chloro-5-iodo, and 2-bromo-3-nitro were classified as highly active. The two dihalogen derivatives were most active in the cucumber germination test and less active in the Acorn squash and the bean spray tests. The 2-bromo-3-nitro compound, on the other hand, was virtually inactive in the cucumber test but was as active as 2,4-D in the bean spray test. The anomalous behavior is noteworthy, since the cucumber germination test is considered more sensitive than the bean spray test. It further emphasizes the necessity for having several test pro-

cedures in a herbicide screening program.

The possibility suggests itself that members of the Cucurbitaceae in general may be tolerant of 2-bromo-3-nitrobenzoic acid; if this is true, then this compound may well find use for controlling dicotyledonous weeds among cultivated members of the family.

None of the disubstituted nonhalogens tested was highly active, but the 2-hydroxy-3-methyl-, 2-methyl-5-nitro-, and the 3,4-dihydroxybenzoic acids were found to be moderately active in one of the three tests.

Of the sixty five halogen-containing trisubstituted compounds tested, only the 2-bromo-3,5-dichloro and the 2-bromo-3-chloro-5-iodo derivatives were highly active. Seven others were classified as moderately active. Of these, five have the 2,3, and 5 positions filled and two the 3,4, and 5.

The nonhalogen trisubstituted derivatives comprise the smallest group tested in this series, yet one out of eleven is highly active. This is 2-nitro-4-methyl-5-hydroxybenzoic acid, which was very inhibitory in the cucumber test but inactive when sprayed upon beans. It is one of the few nonhalogen derivatives of benzoic acid to be reported as a potent inhibitor of plant growth.

Growth responses of kidney beans

During these investigations it was observed that many morphological aberrations induced by substituted benzoic acids were similar in some respects to responses elicited by other growth-regulators. Although these were not studied in great detail, some observations were recorded. Approximately sixty-five compounds were re-examined for ability to induce certain morphological changes. These are recorded in table 3.

Curvature.—One of the earliest observable responses of kidney-bean plants to these growth-regulators is stem curvature (fig. 1). Of the sixty-five selected substituted benzoic acids that were evaluated for this reponse, twenty-five proved to be capable of inducing it. All but one of the twenty-five contained at least one halogen atom. The lone exception is 2-methyl-5-nitrobenzoic acid. This nonhalogen derivative brought about curvature as severe as that induced by its halogen analogs. On the other hand, 2,3,5-triiodobenzoic acid induced only very slight curvature.

Leaf modifications.—Another response frequently seen in plants treated with substituted benzoic acids is leaf modification. In bean plants the gross external appearance of the leaves of treated plants is quite similar to that of plants treated with 2,4-D. The leaves are quite wrinkled and very narrow. The veins are close together, and the lamina does not expand laterally. All compounds that induced stem curvature in beans also induced leaf modifications. In addition, three that failed to cause stem curvature induced formative effects in leaves. These were 2-chloro-3-iodo-5-bromo-, 3,5-dichloro-4-bromo-, and 2,3-diiodo-5-bromobenzoic acid.

One difference between plant responses induced with 2,4-D and with benzoic acid derivatives is their duration. Normally young plants treated with sublethal rates of 2,4-D will produce only three or four malformed leaves before normal leaves appear again. With benzoic compounds the number of malformed leaves is much greater. In one experiment eleven malformed leaves were produced as a result of a spray application with methyl 2-bromo-3-nitrobenzoate. Since primordia for all these leaves did not exist at the time of application, the growth-regulator or its

TABLE 2

ACTIVITIES OF BENZOIC ACID DERIVATIVES ON PRIMARY ROOTS OF GERMINATING CUCUMBER SEEDS (TEST 1) AND ON VEGETATIVE GROWTH OF SEEDLINGS OF ACORN SQUASH (TEST 2) AND RED KIDNEY BEAN (TEST 3)

NAME	TEST		
	1	2	3
Unsubstituted Acid:			
benzoic	I	I	I
Monosubstituted—Halogen:			
2-bromo	H	.....	I
2-chloro	I	.....	I
2-fluoro	I	I	I
2-iodo	I	.....	I
2-iodoso	I	.....	I
3-bromo	I	.....	S
3-chloro	I	.....	I
3-fluoro	I	I	I
3-iodo	I	.....	I
4-bromo	S	.....	I
4-chloro	S.St.	.....	I
4-chloromercuri	S	.....	I
4-fluoro	I	.....	I
4-iodo	I	.....	I
Monosubstituted—Nonhalogen:			
2-acetylamino	Sl.St.	.....	I
2-amino	I	I	I
2-aminophenyl	I	.....	S
2-benzoyl	Sl.St.	.....	I
2-carboxy	S	.....	I
2-carboxymethoxy	I	.....	I
2-(carboxymethylmercapto)	I	.....	S
2-hydroxy	I	Sl.St.	S
2-methoxy	I	I	M
2-methyl	I	.....	I
2-methylamino	.....	.....	I
2-phenoxy	Sl.St.	.....	I
2-phenylureido	I	M	Sl.St.
2-sulfo	S	.....	I
3-acetamido	I	I	I
3-amino	I	I	I
3-carboxy	S	.....	S
3-hydroxy	Sl.St.	.....	I
3-methyl	I	.....	I
3-nitro	I	.....	S
3-sulfo	S	.....	I
4-acetoxy	I	.....	I
4-acetylamino	I	.....	I
4-amino	I	.....	I
4-carbamyl methylamino	I	.....	S
4-carboxy	S	.....	S
4-ethoxy	H.St.	.....	I
4-hydrazino	I	.....	S
4-hydroxy	I	.....	I
4-methoxy	I	.....	I
4-methyl	I	.....	I
4-nitro	S	S	S
4-phenylureido	I	I	Sl.St.
4-ureido	I	I	M
Disubstituted—Halogen:			
2-amino-5-bromo	I	.....	S
2-amino-5-chloro	I	.....	I
2-amino-5-iodo	M.St.	.....	S
2-benzoyl-4-bromo	I	.....	I
2-benzoyl-4-chloro	Sl.St.	.....	I
2-bromo-3-nitro	I	I	H
2-bromo-4-carboxy	S	.....	I
2-bromo-4-nitro	I	.....	I
2,5-dibromo	S	S	M
2-bromo-5-chloro	I	M	S
2-bromo-5-iodo	M	S	S
2-bromo-5-nitro	I	.....	S
2-carboxy-4-bromo	S	.....	I
2-chloro-3-nitro	I	.....	M
2,4-dichloro	I	.....	I
2-chloro-5-bromo	H	M	S
2,5-dichloro	S	S	M
2-chloro-5-iodo	H	I	I
2-chloro-5-methoxy	I	S	I
2-chloro-5-nitro	I	.....	Sl.St.
2-chloro-4-sulfo	I	I	I
2-(3′-chlorophenylureido)-5-bromo	I	I	.....
2-(3′-chlorophenylureido)-5-chloro	I	S	I
2-(3′-chlorophenylureido)-5-iodo	I	I	I
2-fluoro-3-nitro	I	I	I
2-fluoro-5-nitro	I	I	I
2-hydroxy-5-bromo	I	.....	I
2-hydroxy-5-chloro	S	.....	I
2-hydroxy-5-iodo	I	.....	I
2-iodo-3-nitro	I	.....	I
2-iodo-5-bromo	I	I	S
2,5-diiodo	I	.....	I
2-methyl-5-bromo	S	S	I
2-nitro-3-bromo	I	.....	I
2-nitro-3-chloro	I	.....	I
2-nitro-4-bromo	Sl.St.	.....	I
2-nitro-5-chloro	M.St.	.....	I
2-nitro-5-fluoro	Sl.St.	.....	S
2-nitro-6-chloro	I	.....	I
3-bromo-4-amino	I	I	I
3-bromo-4-nitro	I	.....	S
3-bromo-5-nitro	I	.....	Sl.St.
2-nitro-5-bromo	I	.....	I
3,4-dichloro	I	.....	S
3-chloro-4-iodo	I	.....	I
3,4-diiodo	Sl.St.	.....	S
3,5-diiodo	I	.....	I
3-nitro-4-bromo	H.St.	.....	S
3-nitro-4-chloro	H.St.	.....	I
3-nitro-4-fluoro	H.St.	.....	I
3-nitro-4-iodo	M.St.	.....	S
3-nitro-4-iodoso	M.St.	.....	I
Disubstituted—Nonhalogen:			
2-anilino-3-nitro	Sl.St.	.....	I
2-carboxy-3-nitro	I	I	.....
2-hydroxy-3-methyl	I	I	M

TABLE 2—*Continued*

Name	Test		
	1	2	3
Disubstituted—Nonhalogen—Continued:			
2-hydroxy-3-nitro	I	.....	I
2-hydroxymercuri-3-nitro	I	.....	S
2,3-dimethoxy	I	I	I
2-methyl-3-nitro	S	S	I
2-nitro-3-methyl	I	.....	I
2-hydroxy-4-amino	I	.....	I
2-carboxy-4-nitro	I	.....	I
2,4-dihydroxy	I	.....	I
2,6-dihydroxy	S	.....	I
2-nitro-4-arseno	I	.....	I
2-hydroxy-5-amino	I	.....	I
2,5-dihydroxy	I	.....	I
2-hydroxy-5-nitro	I	I	S
2-hydroxy-5-sulfonyl	I	.....	I
2-methyl-5-nitro	I	I	M
2-nitro-4-carboxy	I	.....	Sl.St.
2-nitro-5-methyl	I	.....	I
2,5-dinitro	I	.....	I
2-sulfo-4-nitro	I	.....	I
3,5-dicarboxy	I	.....	I
3,4-diethoxy	I	I	S
3-methoxy-4-hydroxy	M.St.	.....	Sl.St.
3,4-dihydroxy	M	.....	I
3,4-methylenedioxy	I	S	S
3-nitro-4-acetamido	I	.....	Sl.St.
3,4-dinitro	Sl.St.	.....	Sl.St.
3-nitro-4-amino	I	.....	I
3-nitro-4-acetoxy	I	.....	S
3-nitro-4-hydroxy	I	.....	I
3,5-dinitro	I	I	I
Trisubstituted—Halogen:			
2-amino-3,5-dibromo	I	I	M
2-amino-3-bromo-5-chloro	I	.....	S
2-amino-3-bromo-5-iodo	I	.....	I
2-amino-3-chloro-5-bromo	I	.....	I
2-amino-3,5-dichloro	Sl.St.	I	I
2-amino-3-chloro-5-iodo	I	.....	S
2-amino-3-iodo-5-bromo	I	.....	S
2-amino-3-iodo-5-chloro	I	.....	I
2-amino-3,5-diiodo	I	I	I
2,3,5-tribromo	I	I	M
2,3-dibromo-5-chloro	I	S	M
2,3-dibromo-5-iodo	S	S	I
2,5-dibromo-3-chloro	S	S	I
2-bromo-3,5-dichloro	M	M	H
2-bromo-3-chloro-5-iodo	H	.....	M
2,5-dibromo-3-iodo	S	S	I
2-bromo-3-iodo-5-chloro	S	S	S
2-bromo-3,5-diiodo	S	S	S
2-bromo-3,5-dinitro	I	.....	Sl.St.
2-chloro-3,5-dibromo	I	.....	I
2,5-dichloro-3-bromo	I	I	I
2-chloro-3-bromo-5-iodo	S	S	I
2,3-dichloro-5-bromo	I	S	I
2,3,5-trichloro	I	S	S
2,3-dichloro-5-iodo	I	.....	M
2-chloro-3-iodo-5-bromo	S	S	I
2,5-dichloro-3-iodo	S	I	S
2,chloro-3,5-diiodo	S	S	I

Name	Test		
	1	2	3
2-chloro-3,5-dinitro	I	.....	I
2-(3′-chlorophenylureido)-3,5-diiodo	I	I	I
2-(3′-chlorophenylureido)-3,5-dichloro	M.St.	I	S
2-fluoro-3,5-dinitro	I	.....	S
2-hydroxy-3,5-dibromo	H.St.	.....	I
2-hydroxy-3-bromo-5-chloro	I	.....	S
2-hydroxy-3-bromo-5-sulfo	Sl.St.	I	S
2-hydroxy-3,5-dichloro	Sl.St.	.....	I
2-hydroxy-3,5-diiodo	I	I	S
2-hydroxy-3-methyl-5-bromo	S	I	S
2-hydroxy-3-methyl-5-chloro	I	I	M
2-hydroxy-3-methy-5-iodo	I	S	S
2-hydroxy-3-nitro-5-bromo	M.St.	.....	I
2-hydroxy-3-nitro-5-chloro	M.St.	.....	I
2-iodo-3,5-dibromo	I	I	S
2-iodo-3-bromo-5-chloro	I	S	I
2,5-diiodo-3-bromo	I	S	I
2-iodo-3-chloro-5-bromo	I	I	I
2-iodo-3,5-dichloro	I	S	S
2,5-diiodo-3-chloro	I	.....	I
2,3-diiodo-5-bromo	I	S	I
2,3-diiodo-5-chloro	I	I	M
2,3,5-triiodo	I	I	S
3,5-dibromo-4-amino	I	.....	I
3,4,5-tribromo	I	I	I
3,5-dibromo-4-chloro	Sl.St.	I	I
3,5-dibromo-4-hydroxy	I	I	M
3,5-dibromo-4-iodo	I	I	S
3,5-dichloro-4-amino	I	I	S
3,5-dichloro-4-bromo	Sl.St.	M	I
3,4,5-trichloro	Sl.St.	S	I
3,5-dichloro-4-iodo	I	I	I
3,5-diiodo-4-hydroxy	I	.....	I
3,4,5-triiodo	S	.....	I
3,5-dinitro-4-bromo	Sl.St.	I	S
3,5-dinitro-4-chloro	Sl.St.	I	S
3,5-dinitro-4-fluoro	Sl.St.	.....	I
Trisubstituted—Nonhalogen:			
2-amino-3,5-dinitro	I	I	S
2-hydroxy-3,5-dinitro	Sl.St.	I	I
2-hydroxy-3-nitro-5-sulfo	I	.....	I
2-methyl-3,5-dinitro	I	I	.....
2-nitro-4-methyl-5-hydroxy	H	.....	I
2,4,6-trihydroxy	I	.....	I
2,4,6-trinitro	I	.....	I
3,4,5-trihydroxy	I	I	M
3,4,5-trimethoxy	I	.....	I
3,5-dinitro-4-amino	I	.....	S
3,5-dinitro-4-hydroxy	I	.....	Sl.St.
Pentasubstituted—Halogen:			
2-carboxy-3,4,5,6-tetrabromo	Sl.St.	.....	I
2-carboxy-3,4,5,6-tetrachloro	S	.....	I

TABLE 3

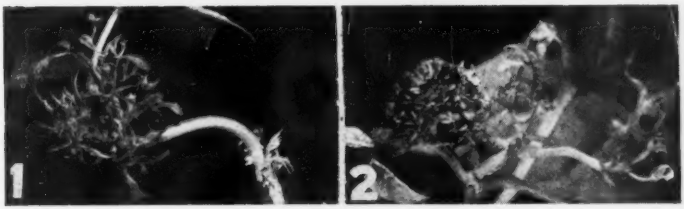
ACTIVITIES OF SUBSTITUTED BENZOIC ACIDS ON KIDNEY BEANS

Ability to induce responses indicated by (+). Where response is positive, the rate listed is the minimum dosage used that was capable of inducing all responses listed. Where response is negative, the rate listed is the highest used in these investigations. All applications were made to the soil (2 lb.) in a 4-inch pot.

Name	Rate in mg.	Curvature	Leaf modifications	Galls	Rosette
Active compounds:					
2-bromo-3-nitro	10	+	+	+	+
2-bromo-3-nitro (methyl ester)	10	+	+	+	+
2,5-dibromo	4	+	+	+	+
2-bromo-5-chloro	4	+	+	+	+
2-bromo-5-iodo	10	+	−	+	−
2-chloro-5-bromo	4	+	+	+	+
2-chloro-5-iodo	4	+	+	+	+
2-chloro-5-iodo (methyl ester)	10	+	+	+	−
2,5-dichloro	4	+	+	+	+
2,5-dichloro (pentachlorophenyl ester)	20	+	+	−	−
2-methyl-5-nitro	20	+	+	+	−
2,3,5-tribromo	4	+	+	+	+
2-bromo-3,5-dichloro	4	+	+	+	+
2,5-dibromo-3-iodo	4	+	+	+	+
2-bromo-3-iodo-5-chloro	4	+	+	−	+
2-bromo-3,5-diiodo	10	+	+	+	+
2,3-dibromo-5-iodo	20	−	.....	+	.....
2,5-dichloro-3-bromo	4	+	+	+	+
2-chloro-3-bromo-5-iodo	10	+	+	+	+
2,3,5-trichloro	20	Slight +	No new leaves	+	−
2-chloro-3-iodo-5-bromo	20	−	+	+	+
2,5-dichloro-3-iodo	10	+	+	+	+
3,5-dichloro-4-bromo	10	−	+	+	+
3,4,5-trichloro	20	+	+	+	−
2-iodo-3-bromo-5-chloro	20	+	+	+	+
2,5-diiodo-3-bromo	10	−	−	Slight	+
2-iodo-3-chloro-5-bromo	10	+	+	+	+
2-iodo-3,5-dichloro	10	+	+	+	+
2,3-diiodo-5-bromo	10	−	+	−	+
2,3,5-triiodo	10	Very slight	+	Slight	+
Inactive compounds:					
benzoic acid	20	−	−	−	−
2-amino	20	−	−	−	−
3-amino	20	−	−	−	−
2-chloro	4	−	−	−	−
3-chloro	4	−	−	−	−
4-fluoro (isopropyl ester)	20	−	−	−	−
2-hydroxy	10	−	−	−	−
2-iodo	4	−	−	−	−
2-methyl	4	−	−	−	−
4-nitro	20	−	−	−	−
2-phenylureido	10	−	−	−	−
4-ureido	10	−	−	−	−
2-amino-5-bromo	4	−	−	−	−
2-amino-5-chloro	4	−	−	−	−
3,4-diethoxy	10	−	−	−	−
3,5-dinitro	10	−	−	−	−
2-fluoro-3-nitro	20	−	−	−	−
2-hydroxy-3-methyl	15	−	−	−	−

TABLE 3—*Continued*

Name	Rate in mg.	Curvature	Leaf modifications	Galls	Rosette
Inactive compounds—Continued:					
2-hydroxy-5-nitro	20	—	—	—	—
2,5-diiodo	4	—	—	—	—
3,4-methylenedioxy	10	—	—	—	—
2-amino-3,5-dibromo	10	—	—	—	—
2-amino-3-bromo-5-chloro	4	—	—	—	—
2-amino-3-chloro-5-bromo	4	—	—	—	—
2-amino-3-chloro-5-iodo	4	—	—	—	—
2-amino-3,5-dinitro	20	—	—	—	—
2-(3′-chlorophenylureido)-3,5-dichloro	10	—	—	—	—
2-hydroxy-3-bromo-5-chloro	4	—	—	—	—
2-hydroxy-3-bromo-5-sulfo	20	—	—	—	—
3,4,5-trihydroxy	20	—	—	—	—
2-hydroxy-3,5-diiodo	10	—	—	—	—
2-hydroxy-3-methyl-5-bromo	20	—	—	—	—
2-hydroxy-3-methyl-5-chloro	20	—	—	—	—
2-hydroxy-3-methyl-5-iodo	20	—	—	—	—
3,5-dibromo-4-hydroxy	20	—	—	—	—
3,5-dinitro-4-bromo	10	—	—	—	—
3,5-dinitro-4-chloro	20	—	—	—	—



FIGS. 1, 2.—Kidney bean. Fig. 1, plant treated with 4 mg. of 2,5-dibromobenzoic acid applied to soil (2 lb.) in 4-inch pot. Note curvature and rosette of malformed leaves on numerous stunted axillary shoots. Fig. 2, terminal gall resembling peach stone in texture on plant treated when 13 inches high with 2-bromo-5-chlorobenzoic acid at rate of 4 mg. per 4-inch pot.

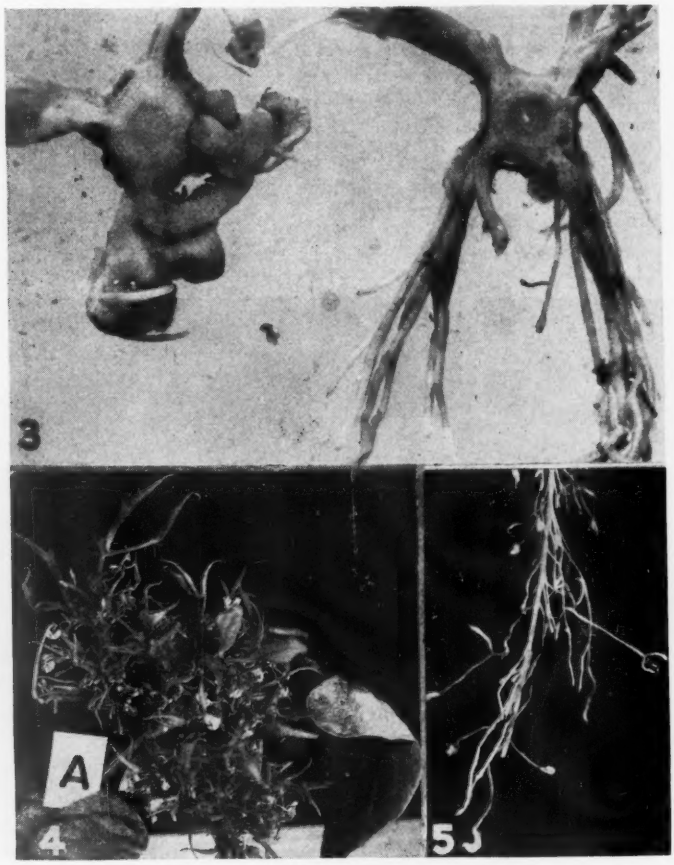
stimulus must have moved into the developing primordia of the younger leaves days to weeks after application. This, then, indicates that the action of many substituted benzoic acids is not immediate and brief, as is claimed for 2,4-D (**2**), but is immediate and prolonged. When applied to a leaf, 2,4-D moves to the apical meristem and is there inactivated by degradation, by union with a cell constituent, or by both methods. The benzoic acid derivatives, on the other hand, move to the same regions but do not appear to be immobilized or degraded so rapidly. They may be translocated to the newer apical meristem as it continues to develop.

GALLS.—Galls also are common morphological changes induced by substituted benzoic acids. However, many of the terminal galls that are induced by these compounds in bean plants continue to grow, resembling peach stones in appearance (fig. 2).

Swellings also appear in the bean hypocotyl and first internode, and under

the proper environmental conditions these develop into fasciated roots (fig. 3). Forty per cent of the compounds were capable of inducing galls or other proliferations.

WITCHES' - BROOM.—The witches'-broom appearance in treated bean plants is fairly common, and approximately twenty-five out of sixty-five benzoic acid derivatives tested were capable of inducing rosetting of this nature. This is quite characteristic of benzoic derivatives, and the loss of apical dominance clearly demonstrates the auxin-inhibiting nature of this chemical structure. Figure 4 illustrates the witches'-broom induced by 2,5-dichloro-3-bromobenzoic acid applied at the rate of 2.5 mg. per pound of soil. Note the numerous small lateral shoots. Over two hundred of these



FIGS. 3–5.—Kidney bean. Fig. 3, cross section of hypocotyl of plant treated with 2,5-dibromobenzoic acid at rate of 2 mg. per lb. of soil. *Left*, at ground level; *right*, halfway between ground and cotyledons. Note fasciation and curvature of roots. Fig. 4, plant showing rosetting and malformed leaves. Seedling was 7 days old when soil was treated with 2,5-dichloro-3-bromobenzoic acid at rate of 2.5 mg. per pound. (Photographed 5 months after application.) Fig. 5, portion of root of plant treated with 2 mg. of 2-chloro-3-bromo-5-iodobenzoic acid per pound of soil. Note swollen root tips.

shoots were counted on one plant. Generally each shoot consists of only one trifoliolate leaf, the leaflets of which are seldom as long as 1 inch.

ROOT-TIP SWELLINGS.—Another response to these compounds is root-tip swellings. These are also induced by phenoxy compounds (7), but the benzoic acid derivatives appear to be a little more potent in this respect. Figure 5 illustrates the root-tip swellings induced by 2-chloro-3-bromo-5-iodobenzoic acid. Generally these swellings are shaped like arrowheads.

GROWTH RESPONSES OF ACORN SQUASH

The responses of Acorn squash plants to substituted benzoic acids differ in many respects from those induced in bean plants. Figures 6, 7, and 8 show the peculiar formative effects induced by

FIGS. 6–8.—Acorn squash. Fig. 6, *left*, plant treated with 5 mg. of 2,5-dichlorobenzoic acid applied to soil in 4-inch pot; *right*, untreated control. Note stunted main axis and failure of leaf blades to develop. Untreated control is in flower. (Photographed 2 months after application.) Fig. 7, close-up of plant treated with 5 mg. of 2,5-dichlorobenzoic acid per 4-inch pot. Note abortive leaf blades. Fig. 8, plant treated with 5 mg. of 2-methyl-5-nitrobenzoic acid per 4-inch pot. Note fusion of leaves and petioles.

2,5-dichlorobenzoic acid and 2-methyl-5-nitrobenzoic acid in Acorn squash.

Stimulation

Although these tests were designed primarily to determine inhibitory activity, a number of compounds actually produced marked stimulation of cucumber roots. Those which have been classified as stimulators are listed in table 4.

From tables 2 and 4 it is evident that in the cucumber test monosubstituted benzoic acids are capable of stimulation and that the substituents contributing to stimulation may be halogen, alkyloxy, or aryl groups, and they may be present in the 2, 3, or 4 positions.

Disubstituted stimulators likewise may have halogen or nonhalogen substituents, and these may be present in virtually every possible position combination.

It is interesting in this regard that at the concentrations employed in these tests (1.0–10 p.p.m.) the following are active stimulators:

2-nitro-3-chloro	M.St.
2-nitro-4-bromo	Sl.St.
2-nitro-5-fluoro	Sl.St.

while the following are inactive as stimulators:

2-nitro-3-bromo	I
2-nitro-5-chloro	I
2-nitro-6-chloro	I

No other halogen-containing disubstituted ortho-nitrobenzoic acids have been tested. Consequently, it is impossible to draw any conclusions from these data except that not all halogens confer activity on the molecule when introduced into a specific position in the benzene nucleus of benzoic acid.

The only complete analogous series of stimulators that was tested was the 3-nitro-4-halogen group, the most active representative of which is the fluorine-containing member. The other halogens occurred in the following order of decreasing activity: Cl > Br > I. The iodoso derivative was slightly more active than the iodo derivatives.

Addition of another nitro group in the 5 position reduces the stimulation of the 3-nitro-4-halogen series, although such trisubstituted compounds still possess activity:

3-nitro-4-bromo	H.St.
3-nitro-4-chloro	H.St.
3-nitro-4-iodo	H.St.
3,5-dinitro-4-bromo	Sl.St.
3,5-dinitro-4-chloro	Sl.St.
3,5-dinitro-4-iodo	Sl.St.

In the 2-hydroxy-5-halogen series the introduction of another halogen or a nitro group into the 3 position confers stimulatory activity to the molecule as measured in the cucumber tests, while the methyl group does not alter the activity of the parent acid. This generalization is not applicable for the iodo series, since all members of this series that were tested are inactive.

Although it cannot be established with certainty from the available data that the mode of action of all these stimulators is the same, nevertheless additional information has been gathered on the most active member of this group. The dosage-response curve of 3-nitro-4-fluorobenzoic acid (fig. 9) indicates that stimulation occurs at concentrations as high as 40 p.p.m. but that inhibition occurs at 100 p.p.m. The maximum stimulation elicited by this compound occurs at 10 p.p.m., where an 80% increase in root length over untreated controls is induced. Histological examination of these roots has been started to determine if the increased root length is the result of unusual cell elongation or cell

division. Results of this investigation will appear in another publication.

One possible mechanism by which the 3-nitro-4-fluorobenzoic acid may stimulate cell elongation is by acting as an auxin and supplementing the activity of the naturally occurring hormone. If this were the case, then small amounts of added indoleacetic acid should also stimulate elongation of intact cucumber roots. This, however, does not occur. Apparently lack of auxin does not limit root growth in this test.

A second possible mechanism for stimulation involves antagonism of naturally occurring auxin that may be present in supraoptimal quantities in normal intact cucumber roots. Although it is true that excised roots require exogenous indoleacetic acid or other growth substance for elongation, evidence is available to indicate that the growth of intact roots is inhibited by exogenous indoleacetic acid (**1**, **2**, **4**). If the mechanism of stimulation is based on antagonism, then the stimulator should be capable of antagonizing an exogenous growth-regulator as well. This actually occurs. Figure 10 illustrates that 3-nitro-4-fluorobenzoic acid can prevent 2,4-D from inhibiting root elongation at a ratio of this compound to 2,4-D of 10:1.

TABLE 4

BENZOIC ACID DERIVATIVES CAPABLE OF STIMULATING ROOT ELONGATION OF GERMINATING CUCUMBER SEEDS

Benzoic acid derivative	Degree of stimulation	Benzoic acid derivative	Degree of stimulation
4-ethoxy	High	3-hydroxy	Slight
3-nitro-4-bromo	High	4-chloro	Slight
3-nitro-4-chloro	High	2-anilino-3-nitro	Slight
3-nitro-4-fluoro	High	2-benzoyl-4-chloro	Slight
2-hydroxy-3,5-dibromo	High	2-nitro-4-bromo	Slight
		2-nitro-4-fluoro	Slight
2-amino-5-iodo	Moderate	3,4-diiodo	Slight
2-nitro-3-chloro	Moderate	3,4-dinitro	Slight
3-nitro-4-iodo	Moderate	2-amino-3,5-dichloro	Slight
3-nitro-4-iodoso	Moderate	2-hydroxy-3,5-dichloro	Slight
2-hydroxy-3-nitro-5-bromo	Moderate	2-hydroxy-3-bromo-5-sulfo	Slight
2-hydroxy-3-nitro-5-chloro	Moderate	2-hydroxy-3,5-dinitro	Slight
3-methoxy-4-hydroxy	Moderate	3,5-dibromo-4-chloro	Slight
2-(3′-chlorophenylureido)-3,5-dichloro	Moderate	3,5-dichloro-4-bromo	Slight
		3,4,5-trichloro	Slight
		3,5-dinitro-4-bromo	Slight
2-acetylamino	Slight	3,5-dinitro-4-chloro	Slight
2-benzoyl	Slight	3,5-dinitro-4-fluoro	Slight
2-phenoxy	Slight	2-carboxy-3,4,5,6-tetrabromo	Slight

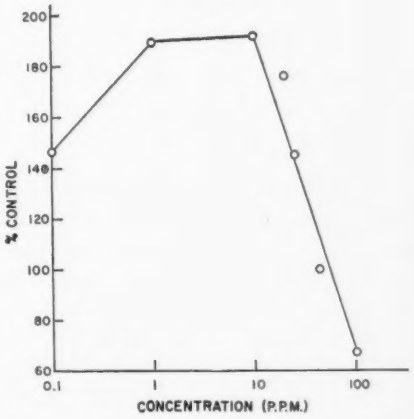


FIG. 9.—Stimulation of cucumber root elongation by 3-nitro-4-fluorobenzoic acid.

Summary

1. More than two hundred substituted benzoic acids were tested for inhibitory activity, but only seven can be classified as highly active.

2. Nonhalogen as well as halogen ring substituents confer activity to the molecule, and active compounds contain substituents in practically all position combinations.

3. Responses induced in Red Kidney bean plants by these compounds are similar in many respects to those induced by phenoxy derivatives and comprise curvature, leaf modifications, galls, witches'-broom, and root-tip swellings. The responses appear to be of longer duration than those induced by phenoxy compounds. It has been suggested that the substituted benzoic acids remain mobile within the plant and continue to migrate to the newly developing leaves at the stem apex. The rate of inactivation within the bean plant appears to be considerably slower than for phenoxy derivatives.

4. The 3-nitro-4-halogen benzoic acids are strong stimulators and can prevent 2,4-D from inhibiting the elongation of intact cucumber roots.

TABLE 5

ACTIVITIES OF SELECTED 2-HYDROXY-5-HALOGEN BENZOIC ACIDS AS AFFECTED BY SUBSTITUENTS IN THE 3 POSITION

Substituent position			Activity (test 1)
2	3	5	
hydroxy	.....	bromo	I
hydroxy	methyl	bromo	I
hydroxy	bromo	bromo	H.St.
hydroxy	nitro	bromo	H.St.
hydroxy	.....	chloro	S
hydroxy	methyl	chloro	I
hydroxy	chloro	chloro	Sl.St.
hydroxy	nitro	chloro	M.St.
hydroxy	.....	iodo	I
hydroxy	methyl	iodo	I
hydroxy	iodo	iodo	I
hydroxy	nitro	iodo	*

* Not tested.

Acknowledgment is made of the assistance of Mr. A. V. MATTIE and Captain D. D. MAXWELL in conducting the tests, Dr. D. BRADBURY in making the histological examination of cucumber roots, and Mr. W. M. NUTTER in preparing the compounds.

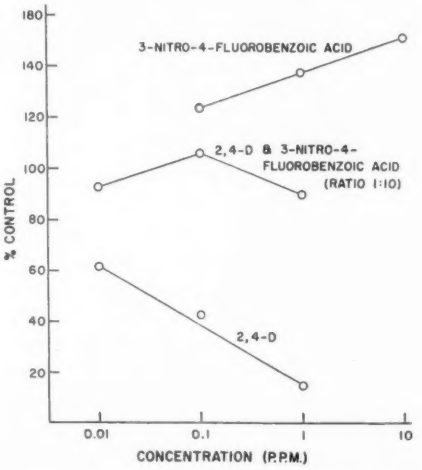


FIG. 10.—Antagonism between 2,4-D and 3-nitro-4-fluorobenzoic acid in cucumber root elongation.

CAMP DETRICK
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EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID AND NAPHTHALENE ACETIC ACID ON INCREASING FRUIT-SETTING AND DELAYING MATURITY OF MONTMORENCY CHERRIES[1]

Q. B. ZIELINSKI AND R. G. GARREN, JR.

Introduction

In preliminary tests on the effectiveness of growth-regulating chemicals in reducing cracking of cherry fruits at maturity, it has been observed that application of 2,4-dichlorophenoxyacetic acid (2,4-D) sprays may result in striking changes in fruit-setting and maturity of Montmorency cherries (*Prunus cerasus*). Results of applications of small quantities, while not indicating an increase in resistance to crack, have suggested possible cumulative effects. From the numerous papers reporting various aspects of plant response to 2,4-D, it is increasingly clear that the period of time over which a plant is exposed to the growth-regulator is of prime importance (**1, 2, 3, 5, 6**). Although no instances have been reported that this growth-regulator may act as an aid in fruit-setting or in delaying fruit maturity, it is significant, as reported by MARTH (**4**), that a mixture of β-indolebutyric acid (0.08%) and 4-chlorophenoxyacetic acid (0.02 %) in lanolin paste was effective in setting lima-bean crosses in the greenhouse. TUKEY and HAMNER (**7**) found striking effects from fall applications of 2,4-D and naphthalene acetic acid (NAA) on delaying blossoming and on the shape of fruits produced the ensuing summer. Unpublished data referred to by TUKEY (**7**) show that vigorous young Montmorency cherry trees may be sprayed in the spring and midsummer with several unspecified growth-regulators at relatively high concentrations with little effect upon the growth or subsequent development of the plant or its parts. On the other hand, it has been found that under Oregon conditions the application of 2,4-D to Montmorency cherry trees in late spring and early summer has resulted in pronounced effects. These are reported here.

Material and methods

In this experiment 2,4-D or NAA sprays at a concentration of 10 p.p.m. were applied during the 1949 growing season at 2-week intervals, beginning June 7 and ending August 19. Spray applications to the same trees were again begun on May 15, 1950, and were repeated at 28-day intervals through August of that year. The 2,4-D formulation used in 1949 and 1950 was the

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regular agricultural Weed-No-More in which bland (paraffin) oil had been substituted for the diesel oil. In 1949 the NAA was applied as an aqueous mixture made from Du Pont's Parmone concentrate. In 1950 both of these sprays were used, and to each Tween 20 (0.05%), manufactured by the Atlas Powder Company, was added as a solublizer. Sprays were applied in 1949 with a Hudson hand-pumped sprayer and in 1950 with a Bean power sprayer which delivered 200 pounds pressure at the nozzle.

Trees selected for these tests were 8 years old, large and vigorous, and making 10–14 inches of new growth annually. Sprays were carefully applied to insure uniform and thorough application to upper as well as lower branches. At each spraying approximately 5 gallons of spray were applied to each tree.

Weather during the 1949 and 1950 periods of application was clear and relatively cool (60°–65° F.) and continued relatively cool for the remainder of the growing season.

Observations were made on leaf-fall, blossoming, tree and fruit growth, and development. Analyses of soluble solids and starch tests were made of fruit and buds from both treated and untreated trees. Measurements were made of growth and development during the growing season as well as final size comparisons at maturity.

Results and discussion

NAA treatment

There was no apparent response of either trees or fruits to the NAA sprays in these tests.

2,4-D treatment

1949.—During the 1949 season the most notable effects of the 2,4-D spray were a delay in maturity and the retention of ripened fruits on the tree throughout the subsequent winter. Although the first spray was applied on June 7, when fruits were still green in color, no differences were apparent until 3 days after the second application on June 21. At this examination several of the younger leaves on each terminal branch were somewhat folded upward, approaching an angle of 90° with the axis of the spur. Occasionally there was a slight twisting of the leaf blades, usually associated with twisted petioles. No effects were noted on leaves on wood of the current season or on older wood, except on one or two of the youngest terminals. Dieback was not in evidence, nor did leaves show necrotic areas or loss of chlorophyll.

Several changes, however, took place in the fruit. Fruits from 2,4-D–treated trees did not maintain a growth rate equal to or show as much red color as those from the check trees. In general, during 1949 fruits of treated trees acquired the characteristic red color 14–21 days later than those of check trees and averaged approximately 18% smaller in volume at full maturity. Not all fruit spurs were affected, however, and there was considerable variation among fascicles on any given spur as to fruit maturity and fruit size.

As the 1949 season progressed, it became obvious that 2,4-D treatments were resulting in retention of fruit and pedicels on the spur. Dried-up fruits were still on the tree during February and March, 1950, and a few persisted into the 1950 growing season.

Fruit buds were collected during December, 1949, for hand sectioning and staining with IKI. Although no quantitative data were recorded, it was obvious that buds from 2,4-D–treated trees were very low in reserve starches,

whereas fruit and leaf buds from untreated trees contained abundant quantities. No tests for sugars were made on this material.

No differences from control trees were noted in the general color of the leaves, in amount of new wood grown, or in date of leaf-fall in any of the treated trees in 1949.

TABLE 1

ANALYSIS OF MATURE MONTMORENCY CHERRY FRUITS IN 1950 FROM TREES SPRAYED WITH 2,4-D DURING SPRING AND SUMMER SEASONS OF 1949 AND 1950

Treatment	Soluble solids (%)	Wt. per fruit (gm.)	Vol. per fruit (ml.)
2,4-D	12.29	2.38	2.35
Control	12.90	4.02	3.83
Percentage decrease		40.8	38.9

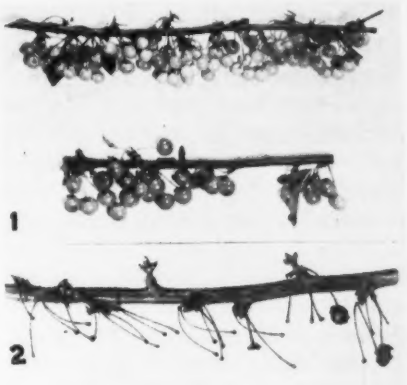
TABLE 2

EFFECT OF 2,4-D SPRAYS DURING SPRING AND SUMMER SEASONS OF 1949 AND 1950 ON FRUIT-SETTING OF MONTMORENCY CHERRY IN 1950

	Treatment		Percentage increase
	2,4-D	Control	
Number fascicles per spur	3.58	2.30	35.9
Number fruits per fascicle	1.68	1.51	10.1

1950.—Trees which received 2,4-D sprays during 1949 blossomed normally and produced a very large crop of flowers in 1950. Although quantitative data on number of blossoms were not recorded at blossoming time, this heavy set of blossoms was apparent later when counts were made on number of fascicles per spur and number of fruits per fascicle (table 2).

During the 1950 season spray applications of 2,4-D or NAA were made at 28-day intervals, beginning on May 15. New shoot growth made prior to spraying appeared to be normal in every respect. Leafing-out occurred as usual, and there was no evidence of dwarfing, curling, or twisting of the leaves. After the second application on June 13, some petiole twisting and curvature of the younger terminal leaves was noted as in the previous season. Older leaves on shoots were not noticeably affected. There has been no evidence of necrosis or dieback in any of the trees.



FIGS. 1, 2.—Montmorency cherry. Fig. 1, *upper*, sprayed with 10 p.p.m. 2,4-D; *lower*, untreated control. Note effect of treatment on fruit size and numbers. (June 30, 1950.) Fig. 2, dormant branch. Effects of 10 p.p.m. 2,4-D on preventing abscission of fruit and pedicels. Seven fruits were lost from this branch while being photographed. (Collected December 27, 1950.)

The analysis of the 1950 crop season with respect to fruit behavior is summarized in table 1. Samples were collected on June 29, when the bulk of the cherry crop was being harvested. On this date fruits on 2,4-D–treated trees were just beginning to show a trace of red color (fig. 1). Data presented (table 1) clearly show the smaller values for soluble solids, weight, and volume of fruit resulting from the

application of 10 p.p.m. of 2,4-D at monthly intervals. Affected fruits gradually became colored, and their sugar content and size increased so as to be commercially acceptable 30–50 days after the normal harvest period. These fruits then remained in an edible and attractive condition until the fall rains started in early September. As was true in 1949, fruits remained on the tree during the following winter of 1950–51 (fig. 2).

The fruit-set in 1950 on 2,4-D–treated trees was unusually heavy. Although no data were recorded, it would appear that the initial fruit-set was heavier than usual. There was a larger number of fruits per fascicle and also a larger number of fascicles per spur on treated trees (table 2). It is apparent that the 1949 spray treatments had a pronounced effect on the differentiation of flower buds. There was no evident injury. On the contrary, the data indicate that there were more individual flower primordia, and these were capable of development into fully expanded flowers and fruits. Thus, these treatments, with refinement, may offer a means of increasing the number of fruits set and of prolonging the harvest season.

Summary

Application of 2,4-dichlorophenoxyacetic acid (2,4-D) at a concentration of 10 p.p.m. to Montmorency cherry trees, semimonthly during the 1949 season and monthly during the 1950 season, resulted in the following effects: (*a*) Delay of 4–6 weeks in fruit maturity; (*b*) no deterioration of commercial quality with the exception of slightly smaller fruit size; (*c*) a larger number of fascicles differentiated in each spur; (*d*) a larger number of fruits per fascicle; (*e*) a smaller amount of starches stored in twigs and buds in winter; (*f*) no evidence of necrosis or dieback or other injury to trees; (*g*) no apparent change in blossoming date or time of petal and leaf-fall; and (*h*) retention of fruits on the tree during the fall and winter season.

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EPIGEAL GERMINATION IN THE LEGUMINOSAE

R. RUGGLES GATES

Introduction

It is well known that in the genus *Phaseolus*, *P. vulgaris* L. is epigeal while *P. multiflorus* Willd. (= *P. coccineus* Lam.) is hypogeal. This difference in the type of germination must depend upon whether the meristematic zone of elongation of the seedling is above or below the point of attachment of the cotyledons. LAMPRECHT (**7, 8**) has found that these two species of *Phaseolus* differ by two main genes as shown by (*a*) epigeal or hypogeal germination and (*b*) the stigmatic surface running down the inner or outer face of the pistil. He also showed that neither of these genes can be transferred to the other species, because, for instance, when the epigeal factor is transferred from *P. vulgaris* to *P. multiflorus*, it produces a gametic lethal (sterile) or a zygotic lethal condition, although the meiotic divisions in the hybrid are said to be fully regular and normal.

Thus *P. vulgaris*, including all varieties of garden bean, is homozygous for epigeal, and *P. multiflorus* (the scarlet runner) for hypogeal, germination. It is reasonable to suppose that in the common ancestry of these two species there were individuals heterozygous for this pair of genes, one condition having originated from the other through a mutation. Apparently *P. multiflorus* is self-sterile (**1**), but *P. vulgaris* is not. A mutation could thus have arisen in the latter, producing intersterility with *P. multiflorus* early in the history of the species, or before they were recognizably different species. The self-sterility mechanism in *P. multiflorus* could have arisen at a later date, after the two species had become more fully differentiated. That intersterility can arise as the result of a single gene mutation is known in the case of *Crepis tectorum* (**3**) and *Hutchinsia alpina* (**10**). Strains of *C. tectorum* crossed with certain other species produce only lethal seedlings, whereas certain strains of *H. alpina* crossed with *H. brevicaulis* produce only empty seeds. There is no necessity to assume isolation as a prerequisite condition. This "gene" mutation might also be a translocation or even an extensive inversion in a chromosome, but in that case there should be some evidence of it in a careful study of pollen meiosis in the F_1 hybrid between the two species. A gene mutation causing intersterility would need to occur repeatedly in order to spread through the population. When such intersterility is once established, the forms involved will continue to diverge through different mutations until they become distinct species.

All mutations apparently recur with a certain frequency, which may be high or low. But why any mutation occurs naturally at a particular time (in a particular cell) remains unexplained, although it is clear that evolution could not take place if *new* mutations, which had never occurred before, did not arise from time to time. The necessity for repeated mutations in evolution has not yet received from geneticists the attention it deserves.

Experimentation and observations

To analyze further the germination conditions in these two species of *Phaseolus*, experiments were made in July, 1949, by planting the seeds of both in different positions and noting the effects on germination. The results are shown in the accompanying photographs made by Mr. FRANK WHITE. In figure 1 the seeds were planted in a pot in the ordinary way and covered with soil, no attention being paid to their position. The four seedlings on the left are *P. multiflorus*, the four on the right *P. vulgaris*. It will be seen that in *P. multiflorus* the base of the primary shoot is always vertical as in *P. vulgaris*, whereas the primary root may at first grow out in any direction. The arch of the hypocotyl is still clearly seen in the seedlings of *P. vulgaris*, the primary root arising from the tip of the hypocotyl and the lateral roots taking their origin from the same point.

In figure 2 are shown five seedlings of *P. multiflorus* in which the seeds had been planted in different positions, as indicated.

It will be seen that while the primary shoot is already vertical in every case, the hypocotyl has taken quite different directions according to the position in which the seed was placed. Evidently the hypocotyl is at first not sensitive to gravity but elongates in whatever direction it happens to be placed. Then the tip becomes sensitive and grows downward, at the same time producing lateral roots as well as a terminal root in *P. multiflorus*, while in *P. vulgaris* practically all the roots arise from the tip of the hypocotyl.

FIG. 1.—Four seedlings on left, *Phaseolus multiflorus;* four on right, *P. vulgaris*

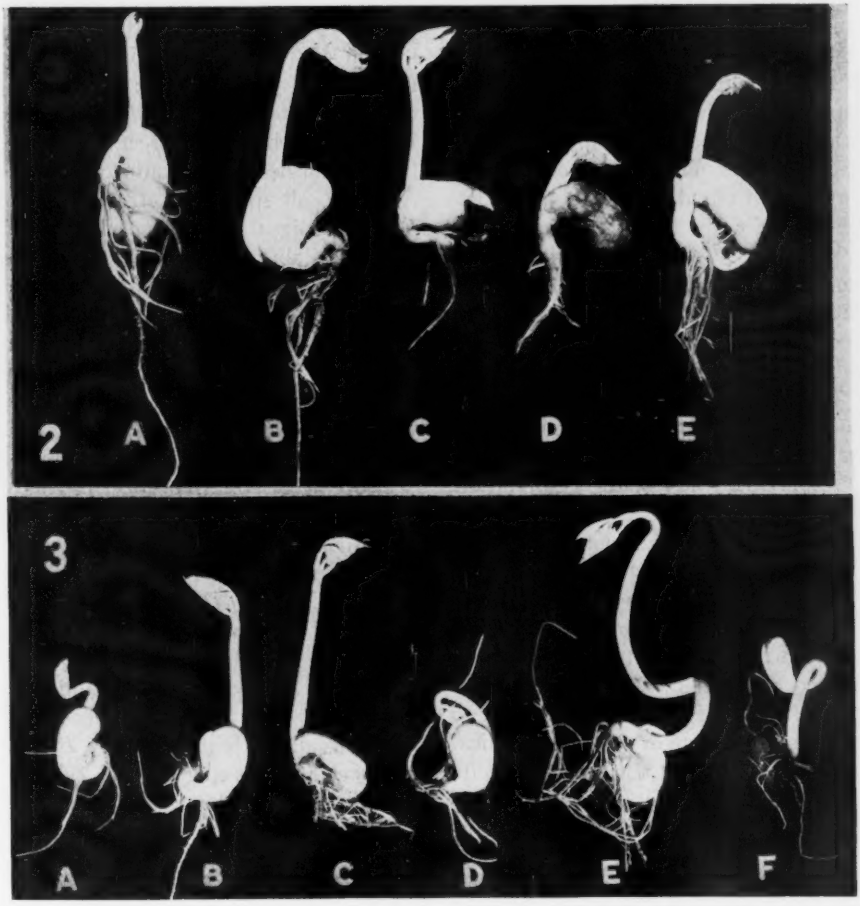
Figure 3 shows five seedlings of *P. multiflorus* and one (*right*) of *P. vulgaris*. The latter seed was planted in a vertical position for comparison with figure 2 (*right*). The position of the seeds in figure 3 was as shown in the legend.

The hypocotyl in *P. multiflorus* apparently never develops geotropic sensitivity, the primary root which develops from

its tip, being, however, positively geotropic, as shown by its direction of growth (figs. 1, 2).

Discussion

The position of the meristematic zone above or below the attachment of the cotyledons is presumably determined by the distribution of auxin. Absence of auxin from the young hypocotyl of *P. multiflorus* would account for its lack of geotropic sensitivity. In experiments with auxin, briefly published by JACOBS (4) since this paper was written, it was found that in kidney-bean seedlings the basal portion of the hypocotyl during the first 3 days of germination contains practically no auxin; this confirms my conclusion that the hypocotyl in *P. multiflorus* also is not geotropically sensitive.



FIGS. 2, 3.—Fig. 2, seedlings of *P. multiflorus* planted with: *A–D*, seed horizontal; *E*, seed vertical. *A*, hilum up; *B*, hilum down; *C*, seed on side; *D*, hilum down (younger). Fig. 3, *A–E*, seedlings of *P. multiflorus* planted with: *A*, seed vertical (young); *B*, seed vertical (older); *C*, seed placed obliquely, side up; *D*, seed horizontal, side up; *E*, seed horizontal, side up (older). *F*, seedling of *P. vulgaris*, seed vertical.

Later, auxin from the cotyledons and epicotyl is transported through this part of the hypocotyl to the tip, which then develops geotropic sensitivity. If the very young seedling is given auxin and sucrose, the basal region of the hypocotyl grows earlier than normally and presumably shows sensitivity to gravity. In further studies JACOBS (**5, 6**) found that in *P. vulgaris* auxin transport downward in the hypocotyl begins in seedlings 3–5 days old and increases to the eighth day, but the auxin remains small in amount during this period. In other experiments with *P. vulgaris*, HEMBERG (**2**) removed the cotyledons from seedlings 10 days old (having one pair of leaves) and cut down the hypocotyl to a length of 5 cm. They were then rooted in aqueous solution. He found that during the first 4–5 days after cutting off the base of the hypocotyl, the increase in free auxin was "inconsiderable," but the bound auxin increased during that time.

The relatively large seeds of the rubber tree, *Hevea brasiliensis* (Euphorbiaceae) are epigeal when germinated in light soil or sand. In a study of the germination, SPRECHER (**11**) found that the hypocotyl normally emerges between the petioles of the cotyledons, e.g., if the seed is placed with the micropyle down. Many anomalies of germination are described, which PETCH ascribed to the position of the seed. SPRECHER was uncertain whether the position of the hypocotyl is due to positive geotropism or to some chemical orientation, but the above experiments with *Phaseolus* make it appear that here too the hypocotyl is not at first geotropically sensitive. Normally a ring of secondary roots develops near the tip of the primary root, but if the latter fails to develop further they become thicker and grow downward, apparently changing from plagiotropic to positively geotropic. SPRECHER (**11**) also showed that the primary shoot, especially in light, may grow for a time in very irregular directions or even like a figure *8*, until a negatively geotropic secondary shoot finally arises from it and grows upward.

In his extensive study of seedlings, LUBBOCK (**9**) showed that the epigeal and hypogeal types of germination are both widely distributed in the Leguminosae. In the following, LUBBOCK's observations have been classified, using the taxonomic system of TAUBERT (**12**) in the *Pflanzenfamilien*. Mimosoideae: The Ingeae are represented by *Pithecolobium saman* Benth., which has an erect hypocotyl, but the cotyledons are caducous. In the Acacieae, all species of *Acacia* are apparently epigeal, but the cotyledons differ remarkably in different species. In *A. delbata* Link they are short, linear-oblong, caducous; in *A. oswaldi* F. Müll. they are obovate-oblong, sagittate at base; in *A. burkittii* F. Müll. broadly triangular and auricled at base; in *A. dodoneaefolia* Willd. connate at base. *Leucaena glauca* Benth. in the Eumimoseae is also epigeal, the hypocotyl having a "corky collar" just above ground level. The Adenanthereae are represented by *Adenanthera pavonina* L., *Prosopis juliflora* DC., and *Dicrostachys cinerea* Wight and Arn., all epigeal.

In the Caesalpinioideae, *Copaifera officinalis* L. (Cynometreae) and *Hymenaea courbaril* L. (Amherstieae) are both epigeal. In the Bauhinieae, *Bauhinea tomentosa* L. is epigeal, in *B. carronii* F. Müll. the cotyledons "show a tendency to become subterranean," while in *B. corymbosa* Roxb. and *B. hookeri* F. Müll. they are "rather fleshy and do not readily unfold to the light." *Cercis siliquastrum* Willd. is epigeal, but the hypocotyl is short (5–6 mm.) and the cotyledons rotund. In the tribe Cassieae, the species

of *Cassia* appear to be all epigeal, but the cotyledons vary much in shape in different species. *Caesalpinia tinctoria* Domb., *Poinciana gilliesii* Hook., and *Haematoxylon campechianum* L., representing the Eucaesalpinieae, are all epigeal, the cotyledons being orbicular in the first, oblong in the second, and deeply bipartite in the third. Relatively little attention has yet been given to the inheritance of cotyledon characters.

Thus it appears that in the two "lower" tribes of Leguminosae (Mimosoideae and Caesalpinioideae) epigeal germination is general, if not universal. If they are ancestral in the order, then it began with epigeal germination, and the hypogeal members of the Papilionatae have probably been derived from epigeal ancestors. In the evolution of the ten subtribes of Papilionatae the transition between epigeal and hypogeal germination must have occurred a number of times.

All the Sophoreae examined by LUBBOCK were hypogeal. In *Sophora secundiflora* Lag. the hypocotyl was undeveloped, and the cotyledons remained underground with their faces closely appressed; in *Edwardsia chilensis* Miers the hypocotyl is subterranean, the fleshy cotyledons also remaining underground; in *Castanospermum australe* A. Cunn. the enormous cotyledons are subterranean, and the hypocotyl is undeveloped. In the Podalyrieae, on the contrary, the four species germinated, *Chorizema cordifolium* F. Müll., *C. ilicifolium*, *Podalyria australis* Vent., and *Viminaria dentata* Sm., were all epigeal, *Podalyria* having a short hypocotyl (3–4 mm.) with oblong, obtuse cotyledons, while *Viminaria* had sessile, linear cotyledons. The Genisteae also are epigeal. Species of *Bossiaea*, *Hovea*, *Goodia*, *Crotalaria*, *Lupinus*, *Laburnum*, *Genista*, and *Ulex* were germinated. The hypocotyl ranged in length from 2–3 mm. in *B. rufa* R. Br. to 2–2.5 cm. in *Lupinus sulphureus* Doug.

In the Trifolieae the results are not so clear. *Ononis natrix* DC. has a subterranean hypocotyl. In *Trigonella corniculata* L. the hypocotyl is "herbaceous," 2 cm. long; in *T. foenum-graecum* L., "much stouter"; and in *T. gladiata* Sm., "subterranean." In *Medicago orbicularis* All. the seeds germinate within the coiled legume, and the seedlings are clearly epigeal. *Melilotus officinalis* Willd. and *M. leucantha* Koch are both epigeal. In *Trifolium boissieri* Guss. the hypocotyl is colorless below and purple above, while the cotyledons are only 8 mm. long but deep green. *T. subterraneum* L. is evidently also epigeal. Its name refers to the geocarpic fruits, which are also formed in *Trigonella aschersoniana* Urb. as well as in the well-known *Arachis hypogaea* L. and *Voandzia subterranea* Thouars.

In the Loteae, *Lotus tetragonolobus* L. has an erect hypocotyl which is "partly subterranean." The Galegeae appear to to be mostly epigeal, but in *Galega officinalis* L. the hypocotyl is "mostly subterranean." In *Indigofera australis* Willd. the hypocotyl is only 8–12 mm. long, and the cotyledons are caducous. *Robinia pseudo-acacia* L., *Carmichaelia pilosa* Col., *Astragalus sulcatus* L., *A. thianschanicus* Regel, and *Sesbania* spp. are clearly epigeal. The Hedysareae are apparently all epigeal. In *Hedysarum denticulatum* Regel the hypocotyl in germination pierces the lower valve of the pericarp, while in *H. cononarium* L. the very short hypocotyl emerges between the valves, and the cotyledons are at the ground level. In *H. capitatum* Desf. the hypocotyl is "erect or decumbent," 8–20 mm. above ground, emerging between the halves of the seed pod. *Aeschynomene aspera* L. is also epigeal. In *Desmodium*

canadense DC. the radicle emerges at the dorsal suture of the fruit or breaks through the side walls. After fixing itself in the ground, the lengthening hypocotyl "generally carries the lomentum and seed up with it." *Lourea vespertilionis* Desv. is also epigeal, but the hypocotyl is only 2–4 mm. above the soil, and in *Lespedeza angustifolia* Ell. it is also very short.

In the tribe Dalbergieae, *Dalbergia hupeana* Hance, *Pterocarpus rohrii* Vahl, and *Lonchocarpus latifolius* H.B.K. are all epigeal. In the Vicieae the genus *Lathyrus* is hypogeal, *L. nissolia* L. having a very short hypocotyl and subterranean cotyledons, *L. articulatus* L. a hypocotyl undeveloped or scarcely elongated, and *L. aphaca* L. an undeveloped hypocotyl with subterranean cotyledons.

Finally, in the Phaseoleae conditions are clearly mixed. *Clitoria ternatea* L. is epigeal, with a hypocotyl 3–4 cm. long, as is *Pueraria thunbergiana* Benth. *Pachyrrhizus angulatus* Rich., *Atylosia candollei* W. & Arn., *Rhynchosia viscosa* DC., and *Flemingia grahamiana* W. & Arn., on the other hand, are hypogeal, the hypocotyl undeveloped or none, and the cotyledons subterranean, while in *Galactia glabella* Michx. the cotyledons are "just emerging from the soil."

In the genus *Erythrina*, *E. suberosa* Roxb. and *E. vespertilio* Benth. have an undeveloped or very short hypocotyl, the fleshy cotyledons remaining in the testa underground, while in *E. monosperma* Lam. the hypocotyl is erect, pale green, 7.8 cm. long. In the genus *Vigna*, *V. lutea* A. Gray and *V. vexillata* Benth. have no hypocotyl and subterranean cotyledons. In *Phaseolus*, LUBBOCK found that *P. vulgaris* was epigeal with a hypocotyl 7 cm. long, but *P. multiflorus* was not germinated.

In some related species the difference between hypogeal and epigeal germination can hardly depend on a single gene, but *Erythrina monosperma* (epigeal) may form a pair with *E. suberosa* or *E. vespertilio* similar to that in *Phaseolus*. It is evident that great differences also exist in such features as the length of the hypocotyl and the size, shape, and general character of the cotyledons in different species of the same genus.

From these results it appears that the tribes are for the most part either hypogeal or epigeal throughout. All the Mimosoideae and Caesalpinioideae appear to be epigeal, but in the Papilionatae the Podalyrieae, Genisteae, Galegeae (mostly), Hedysareae, and Dalbergieae are epigeal, the Sophoreae and Vicieae hypogeal, the Loteae partly hypogeal, while the Trifolieae and the Phaseoleae are divided between hypogeal and epigeal germination. These relations appear to strengthen the case for regarding the Papilionaceae with their highly specialized floral structure as a separate family in which hypogeal germination was the primitive condition, or else they are evolving back from epigeal to hypogeal conditions. It appears clear, however, that in the evolution of the Papilionatae the transition between epigeal and hypogeal germination has happened several times. The more or less intermediate conditions with short hypocotyl in certain species indicate that in these cases the transition probably involved not one but several mutations.

Summary

1. Epigeal and hypogeal germination in *Phaseolus vulgaris* and *P. multiflorus*, respectively, constitute a pair of interspecific genes. By germinating the seeds of the garden bean and the scarlet runner (a pole bean) in various positions, it was found that the hypocotyl of the latter is not at first geotropically sensitive but

may grow out in any direction. This is supported by the recent finding that the hypocotyl in seedlings of *P. vulgaris* does not contain diffusible auxin. On the other hand, the primary root which develops from the tip of the hypocotyl is always positively geotropic. There is no evidence as to why the zone of growth is above the attachment of the cotyledons in one species and below it in the other, but distribution of auxin in the very young seedlings is presumably involved.

2. A survey of the Leguminosae as regards the methods of germination shows that the Mimosoideae and Caesalpinioideae are apparently all epigeal, whereas in the Papilionatae some tribes are epigeal, some hypogeal, both types of germination being found within the Trifolieae and Phaseoleae. In the evolution of the order the transition from one type to the other must have occurred several times. *Erythrina monosperma* and *E. suberosa* may form a pair of species like *P. vulgaris* and *P. multiflorus*, but wide variation in length of hypocotyl in different species and other intermediate conditions indicate that in some cases several genes are probably involved in the transition. There is need for much work on the genetics of cotyledon size and shape in the Leguminosae.

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A NEW AMERICAN SPECIES OF BOWMANITES[1]

HENRY N. ANDREWS, JR., AND SERGIUS H. MAMAY[2]

Introduction

The genus *Bowmanites*, which includes a rather varied assemblage of cones believed to have been borne by Sphenophyllalean plants, was monographed by HOSKINS and CROSS in 1943 (3). The following year ARNOLD (1) described a heterosporous species from the Michigan coal basin, although more recently he has removed this to the genus *Discinites* (2: 220). A detailed review of the genus is embodied in those publications.

In the present account we shall describe a species recently found in an Illinois coal ball; it represents morphological characteristics that indicate a new specific entity and is particularly remarkable for its very minute size, being considerably smaller than most of the previously reported species.

Our specimen was found in a coal ball from Berryville, Illinois, at an outcrop (9) which lies in the upper part of the McLeansboro group (upper Pennsylvanian).

Observations

BOWMANITES BIFURCATUS SP. NOV.

The specimen described under this new binomial is a minute cone only slightly more than 3 mm. in diameter (fig. 2). About 1.5 cm. of its length was preserved, including the terminal portion. The distance between nodes is about 0.8 mm., there being some seventeen whorls of appendages present. A whorl consists of six bracts, above which are borne six pairs of sporangia. Each sporangium is attached at its distal end to a recurved forking sporangiophore. All but the three uppermost whorls are fertile.

An oblique transverse section was exposed by the initial saw cut. Subsequent cuts were made in order to prepare transverse and longitudinal series of peel preparations. Aside from the basal third of the cone, which was badly crushed, the entire specimen was used in the preparation of these peels, which were mounted and catalogued under the numbers 1740–1861, inclusive, in the paleobotanical slide collection of the Henry Shaw School of Botany.

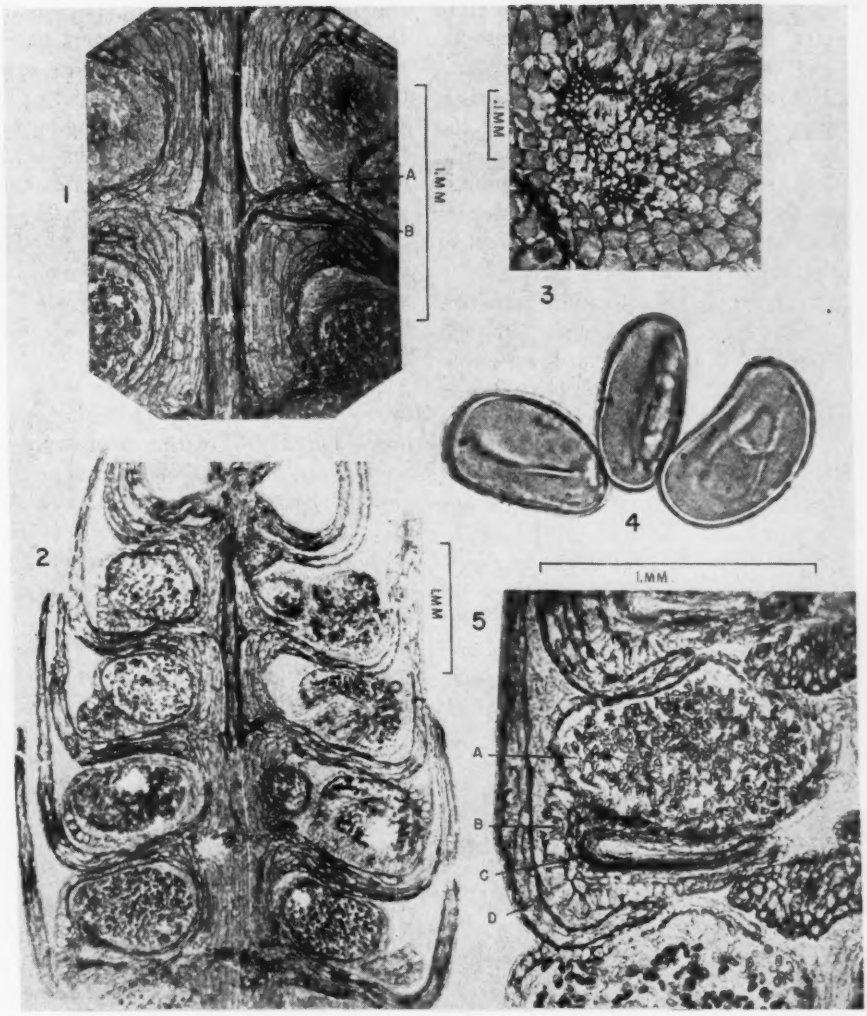
AXIS

The cone axis is fairly stout and, when seen in a typical internodal transverse section, displays an essentially terete outline. The cells of the epidermis are relatively small, with the cortical cells increasing in size toward the vascular system. There is no apparent cuticularization of the epidermis or specialization of cortical tissues. The greatest diameter of the axis (at the median internodal position) is about 0.75 mm.

The vascular system of the axis (fig. 3) has the triarch structure typical of the Sphenophyllales. It consists of three isolated strands of protoxylem elements

[1] A study aided by a grant from the American Philosophical Society.

[2] John Simon Guggenheim Fellow, University of Cambridge, England, 1950–51.



FIGS. 1-5.—*Bowmanites bifurcatus*. Fig. 1, median longitudinal section through cone, including parts of three nodes. At *A* and *B*, respectively, initial branches of appendage trace pass out into (*A*) sporangiophore and into (*B*) sporophyll. Slide No. 1826. Fig. 2, median longitudinal section through upper part of cone, showing general organization. Slide No. 1828. Fig. 3, transverse section of central portion of axis, showing three strands which compose stele. Slide No. 1793. Fig. 4, three spores from sporangium on slide No. 1804. Spores measure approximately 20×40 μ. Fig. 5, longitudinal section, showing *A*, sporangium; *B*, sporangiophore; *C*, trace of sporangiophore; *D*, sporophyll. Slide No. 1815.

situated at the angles of an equilateral triangle. Each strand contains eight to twelve tracheids, whose diameters do not exceed 12 μ.

The longitudinal aspect of the axis is best seen in figure 2, where two of the three vascular strands appear. The individual tracheids, under high magnification, show spiral and annular thickenings. There is no evidence of metaxylem elements.

It is of interest that the apical meristem of the specimen is preserved. Although the tip ends of the terminal bracts were lost in grinding, the greater portion was kept intact. The apical meristem appears as a broadly conical structure approximately 0.28 mm. in basal diameter and 0.16 mm. in length. At either side are seen two slightly swollen loci which are interpreted as the youngest sporophyll primordia. Immediately below these lie two well-developed but small bracts. The cells of the outer layer of the apex appear to be slightly elongated in the anticlinal direction, presenting somewhat of a palisade aspect.

Appendages

The appendages are arranged in superposed verticels, each consisting of six units. Each unit is composed of a lower (abaxial) sterile portion—the bract—and a fertile upper (adaxial) portion—the sporangiophore (figs. 2, 5).

Bracts.—The basal portions of the bracts are fused into a disklike collar which extends perpendicularly or in a slightly depressed direction from the cone axis.

The horizontal or fused portion of the bracts extends about 1 mm. from the axis; beyond this the bracts separate from one another and assume an ascending position roughly parallel to the axis (fig. 2). The free, ascending portions of the bracts attain lengths of 2.5 mm. and thus overlap those of the immediately superior whorl, extending in some cases beyond the second superior node.

The base of the free ascending part of the bract increases abruptly in width, resulting in a "winged" appearance when they are viewed transversely at this point (fig. 10). The margins of adjacent bracts overlap each other, but it has not been possible to determine whether this imbrication occurs in any regular fashion.

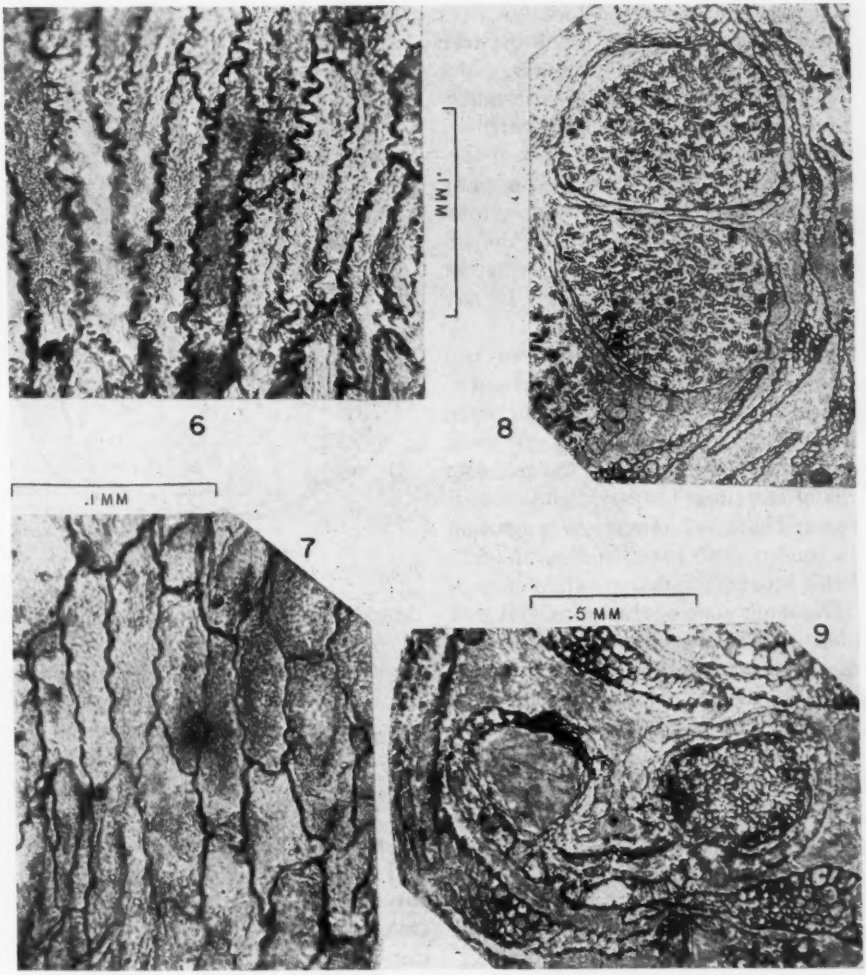
A distinctive feature of the bracts is the bifurcation of the free ascending portions. This occurs within a distance of less than the length of one internode and may be observed in figures 8 and 10. Although crushing and distortion have prevented a clear count of the total number of bract segments in any single transverse section, twelve appears to be the maximum number, and there is no evidence that the bract was divided beyond the primary bifurcation.

The nature of the epidermal cells of the bracts is a point of particular interest. They average about 170 μ in length and 30 μ in width, and their lateral walls present a characteristic sinuous outline (fig. 6). The only other cells of this general type that have been observed are those composing the epidermis of the sporangia (fig. 7), although there the degree of sinuosity is not nearly so pronounced.

Sporangiophores.—The disk of each verticel subtends six sporangiophores, one corresponding to each segment, or bract. The point of departure of the sporangiophore is nearly axillary, i.e., it actually departs from the upper surface of the bract but close to the axis. The sporangiophores are slightly flattened in the plane of the disk, measure about 0.25

mm. in greatest diameter, and attain a length of about 1.0 mm.

Through the greater portion of its length the sporangiophore lies closely appressed to the upper surface of the subtending disk (fig. 5), but at the distal end it assumes a sharply ascending position (fig. 2) and undergoes a bifurcation, each branch terminating in a single sporangium. Figure 9, a tangential section through the distal portion, illustrates this feature very clearly. The two sporangia are shown in connection with the sporangiophore whose vascular bundle



FIGS. 6–9.—*Bowmanites bifurcatus*. Fig. 6, surface view of outer epidermis of sporophyll. Slide No. 1851. Fig. 7, surface view of epidermal cells of sporangium. Slide No. 1831. Fig. 8, transverse section through pair of sporangia, showing subtending bract. Slide No. 1797. Fig. 9, tangential section through outer part of cone, showing bifurcation of sporangiophore and two sporangia. Slide No. 1856.

has divided into two parts, one directed toward each sporangium.

SPORANGIA.—Although they are generally somewhat distorted by mutual appression and to some extent flattened by the pressure imposed by the disks both above and below, the sporangia are ovoid in shape, with the elongation in the radial direction. The sporangia measure from 1.10 to 1.20 mm. in length and between 0.55 and 0.60 mm. in diameter. Since each bract subtends a single sporangiophore, which, in turn, is terminated by two sporangia, the total number of sporangia in a single whorl is normally twelve. In slide no. 1750, where the total number of sporangia is twelve, the extremely crowded condition suggests that this number was not exceeded in any single whorl.

The sporangial wall consists of two cell zones. The outer zone is composed of a single layer of large cells with thickened and slightly sinuous walls (fig. 7); these resemble to a certain extent the annulate cells of certain other pteridophytic sporangia. There is, however, no suggestion of a true annulate function, since the cells of this layer are rather constant in size.

The inner zone of the sporangial wall is best seen at the base of the sporangium (fig. 9), where it is shown to be an extension of the undifferentiated tissues of the sporangiophore into the sporangial base, forming a shallow, saucer-like aggregate of cells. The individual cells of this tissue are so small and thin walled that they are distinguished only with difficulty. The outer zone of the sporangial wall extends downward from the sporangial base and continues along the lower and lateral surfaces of the sporangiophore (fig. 5); the upper surface remains quite undifferentiated.

SPORES.—Spores are abundantly preserved in nearly every sporangium. Numerous closely associated dyad-like pairs as well as occasional intact tetrads are present in some of the spore masses; but, judging from the mode of spore development in the archegoniates as described by KNOX (5), it seems clear that the spores were essentially mature at the time of fossilization.

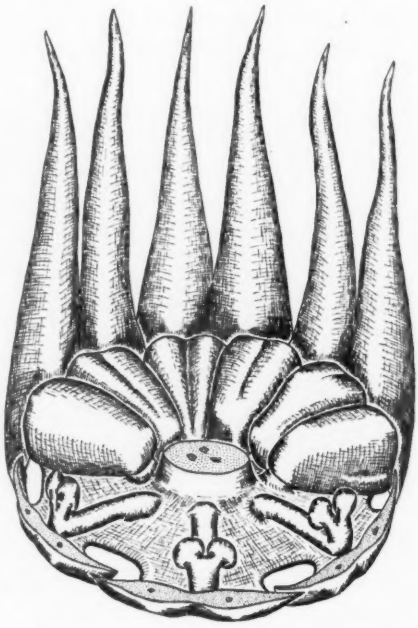


FIG. 10.—Reconstruction of one node of cone of *Bowmanites bifurcatus*. Sporangia and bracts have been removed in foreground in order to show orientation of sporangiophores.

The spores are of the bilateral-monolete type (fig. 4). In general outline they are reniform and attain lengths of 40 μ and widths of 20 μ. A single longitudinal suture is sometimes apparent on the concave face of the spore wall. The latter displays no apparent ornamentation. In spore morphology alone, this fructification appears to constitute a distinct new species of *Bowmanites*.

Although spores of this type are quite

common among Carboniferous plants, certain significance may be attached to their presence here in a *Bowmanites* fructification. HOSKINS and CROSS (3: 139) have indicated that the genus may be defined partially on the basis of spore morphology: "Spores usually with a ridged (falsely spinose) perisporium; exine pustulose with triradiate tetrad scar extending less than half way to margins; intine delicate."

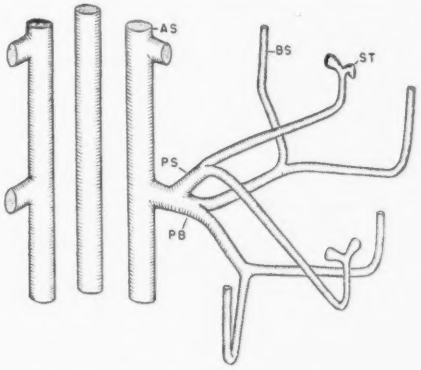


FIG. 11.—Reconstruction of vascular system of *Bowmanites bifurcatus*, showing three central strands with one of three branches given off at each node. Proportions of this unit have been altered somewhat in order to conserve space. *AS*, axial strand; *PS*, primary sporangiophore trace; *ST*, bifurcated end of sporangiophore trace; *PB*, primary bract trace; *BS*, trace to bract segment.

Although there seems to be no question that the spores of all the species of *Bowmanites* known at the time of HOSKINS and CROSS's publication conformed to that portion of the generic diagnosis quoted above, some modification appears necessary in view of the data presented by the specimen described here.

VASCULAR ANATOMY

As pointed out in a preceding paragraph, the vascular system of the cone axis consists of three tiny isolated strands which represent protoxylem elements (fig. 3).

The nodal anatomy is rather simple. A small trace departs from each of the axial strands, the angle of departure being about 90° (figs. 1, 2). Almost immediately after its departure from the stele, the trace divides, the upper branch functioning as a "primary sporangiophore trace" and the lower as a "primary bract trace." Both strands then bifurcate in the horizontal plane; each of the two branches of the upper trace supplies a sporangiophore and each branch of the lower one supplies a sporophyll (fig. 11). Each sporophyll bundle bifurcates in accordance with the bifurcation of the bract.

DIAGNOSIS

Bowmanites bifurcatus Andrews and Mamay, a small homosporous cone of about 3 mm. diameter; six bracts in each whorl fused to form disk at base, free portion sharply upturned and once forked, epidermal cells with strongly sinuous walls; six sporangiophores in each whorl, each sporangiophore forked at tip, bearing two sporangia, epidermal cell walls of sporangia slightly sinuous; spores monolete, smooth walled, averaging 40 × 20 μ.

Locality: Brian farm, Berryville, Illinois.

Horizon: upper part of McLeansboro group, upper Pennsylvanian.

Type specimen: description is based on specimen which was used entirely in the preparation of transverse and longitudinal series of peels which were mounted and preserved under the numbers 1740–1861 in the paleobotanical slide collection of the Henry Shaw School of Botany, Washington University.

Discussion

Discoveries of the past few decades have made striking additions to our knowledge of the cones of the articulates. The diversity of the group in Carboniferous times is evident from the wide range in size and morphology of these organs, and to the taxonomically minded paleobotanist the situation becomes both more fascinating and more chaotic.

In contrast to *Equisetum*—the single living genus of articulates—we find in the Carboniferous a wide array of plants which are particularly striking in the diversity of morphology of their cones. The contrast in size between species such as *Cheirostrobus pettycurensis* Scott and *B. bifurcatus* is no less striking than the differences in their construction. The unique nature of many members of the group is further attested by examples such as *B.* (*Sphenophyllum*) *fertilis* (6) and *Sphenostrobus thompsonii* (7). Although we can only guess at the interrelationships of some of these forms, one can hardly say that our knowledge of the articulates is stagnating!

A few final comments may be added as corrections to the present status of our knowledge of *Bowmanites* and as possible points to be considered by future contributors.

In 1944 ARNOLD (1) described a heterosporous species of *Bowmanites*, whereas all previous members of the genus were considered to be homosporous (3). More recently, however, this supposed heterosporous species has been transferred to *Discinites* (2).

The only way in which *B. bifurcatus* fails to comply with the generic concept as given by HOSKINS and CROSS lies in the morphology of the spores, their monolete, smooth-walled nature presenting somewhat of a contrast to those of previously described species. We are inclined to believe that spores are not likely to present dependable generic characters in all cases. KNOX (4:464) arrived at this general conclusion in her study of pteridophytic spores: "With few exceptions there is little evidence that the more detailed features of spore architecture remain constant within the limits of a family, and even for genera there are not many examples where spore characters could be held to be diagnostic." The wide range in morphology for the spores of *Botryopteris* and *Scolecopteris*, as recently described by MAMAY (8), lends support to KNOX's conclusions.

The general organization of the sterile and fertile appendages of *Bowmanites* cones appears to present the most reliable generic character. Even here, however, the propriety of retaining so diverse an assemblage within one genus may be questioned. As a striking example we may point out the contrast in organization between *B. fertilis* [as described by LECLERCQ (6)] and several other species, such as *B. trisporangiatus*. We would also suggest that several of the species (based on nonpetrified species) included by HOSKINS and CROSS are not sufficiently well known to lend assurance of the validity of their present classification. Their excellent monographic treatment of the group is, nevertheless, a significant step forward, as we are particularly in need of studies which will correlate compressions and petrified fossil remains.

Summary

A well-preserved *Bowmanites* cone is described from the McLeansboro group (upper Pennsylvanian) of Berryville, Illinois. The fossil is a small homosporous cone, about 3 mm. in diameter, and consists of seventeen whorls of appendages. Each whorl consists of six bracts, above which are borne six pairs of sporangia,

each attached at its distal end to a recurved forking sporangiophore. The vascular system of the axis is a small triarch structure. The epidermal cell walls of the bracts are strongly sinuous and those of the sporangia slightly so. The spores are monolete and smooth walled.

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SMALL PETRIFIED SEEDS FROM THE PENNSYLVANIAN OF ILLINOIS

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Introduction

Structurally preserved gymnospermous seeds in the Pennsylvanian deposits of Illinois were first reported by NOÉ (**24**) after coal balls had been obtained in 1922 near Harrisburg and soon afterward at Calhoun and Danville. The aggregation of Paleozoic seeds which now have been described from Illinois conforms to the same system of classification suggested by SEWARD (**30**) for European forms, in which unattached gymnospermous seeds are arranged into three groups—the Lagenostomales, the Trigonocarpales, and the Cardiocarpales.

Among the Lagenostomales of Illinois, KRICK (**21**) and REED (**29**) have reported *Conostoma oblongum*, which was originally described from the Lower Coal Measures of Great Britain. Two new American species, *C. platyspermum* and *C. quadratum*, also have been described in the coal flora of Illinois by GRAHAM (**13**). In addition, *Lagenostoma lomaxi*, a European species, was included by NOÉ (**25**) in an enumeration of petrifaction species from Illinois, and *Lagenostoma* was also mentioned by DARRAH (**9**) in a similar listing for Iowa. No further description, however, has been given of the occurrence of *Lagenostoma* in the American fossil flora.

Several large, structurally preserved seeds from Illinois have been assigned to genera of the Trigonocarpales. From the original coal-ball locality at Harrisburg, KRICK (**21**) has reported *Trigonocarpon pusillus*, *T. parkinsoni*,[1] and *Stephanospermum akenioides*. DEEVERS

[1] After examination of these specimens of *Trigonocarpon*, HOSKINS and CROSS (**17**) did not assign them to described species of the revised genus *Pachytesta*.

(10) has described *Sarcospermum ovale* and ARNOLD and STEIDTMANN (3), *Rotodontiospermum illinoense*. More recently STEWART (31) has described *Pachytesta hexangulata*.

Although many seeds of the Cardiocarpales, existing in the form of impressions, compressions, and casts, have been assigned to the genera *Cardiocarpus* (or *Cardiocarpon*), *Cordaicarpus*, and *Samaropsis*, only the new species of GRAHAM (14), *Cardiocarpus spinatus*, has been described as a petrified seed of the Cardiocarpales in Illinois.

This distribution of species within the three seed orders is more an indication that the structure of the larger Trigonocarpales is often better preserved and is more readily studied than that the Trigonocarpales were more numerous than the smaller seed forms of the Lagenostomales and the Cardiocarpales.

Additional, well-preserved seeds have been found in coal balls collected at the New Delta locality in Williamson County and at the Berryville locality in Lawrence County, Illinois. Using the peel technique, an anatomical study of these new specimens indicates the occurrence of at least two new species of small petrified seeds: *Taxospermum undulatum* and *Coronostoma quadrivasatum*. It is possible, moreover, to clarify the description of *Conostoma quadratum* Graham, one of the species which had previously been described from Illinois.

Observations and discussion

TAXOSPERMUM UNDULATUM SP. NOV.

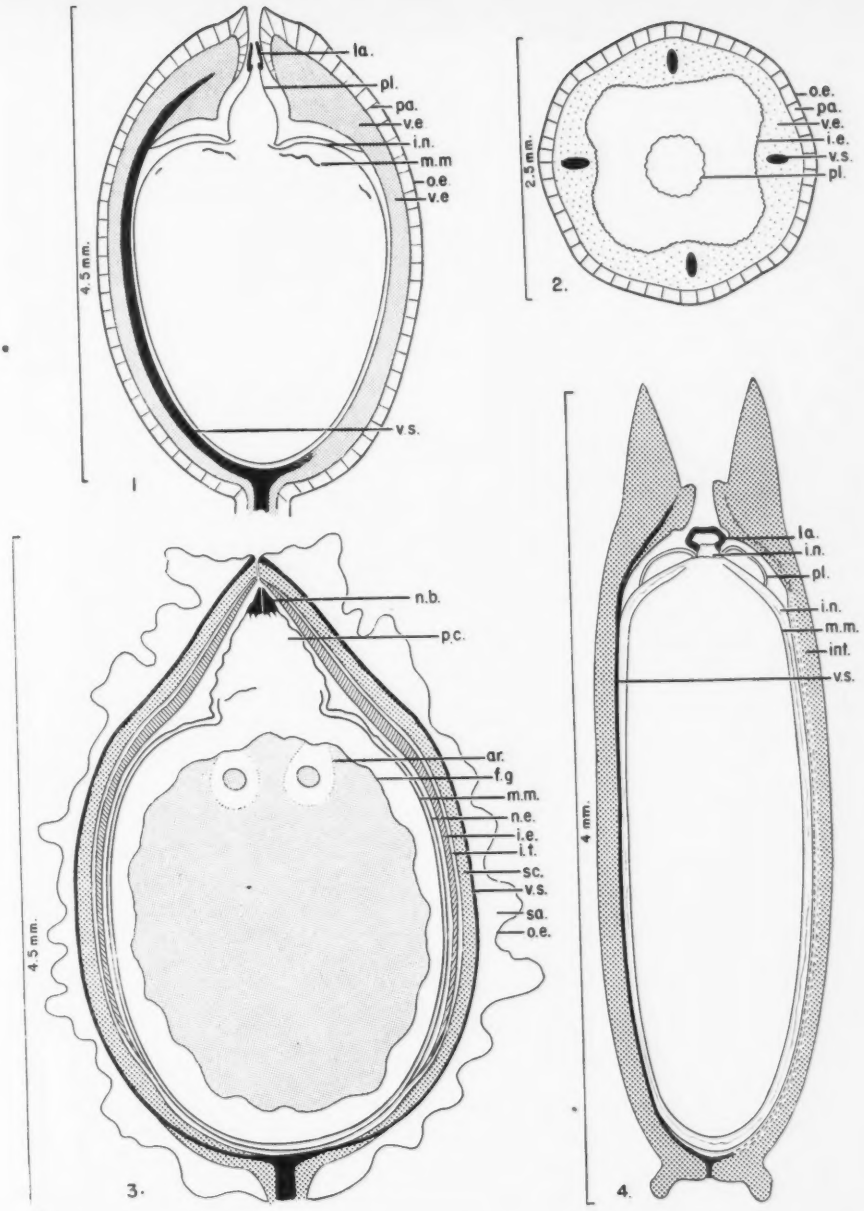
GENERAL STRUCTURE.—Over forty specimens of these small seeds were found in coal balls which were associated with the no. 6 coal at the top of the Carbondale group in the Pennsylvanian system at the New Delta locality in Williamson County, Illinois.

The ovoid seeds (figs. 3, 5) are irregular in outline, measuring 4.5 mm. in length × 3.5 mm. in maximum diameter. The seeds are almost round in transverse section and have only slight lateral flattening. Although the position of each of the two lateral vascular strands (fig. 12) is marked by a blunt sclerotestal rib, there is no evidence of corresponding wings in the irregular sarcotesta.

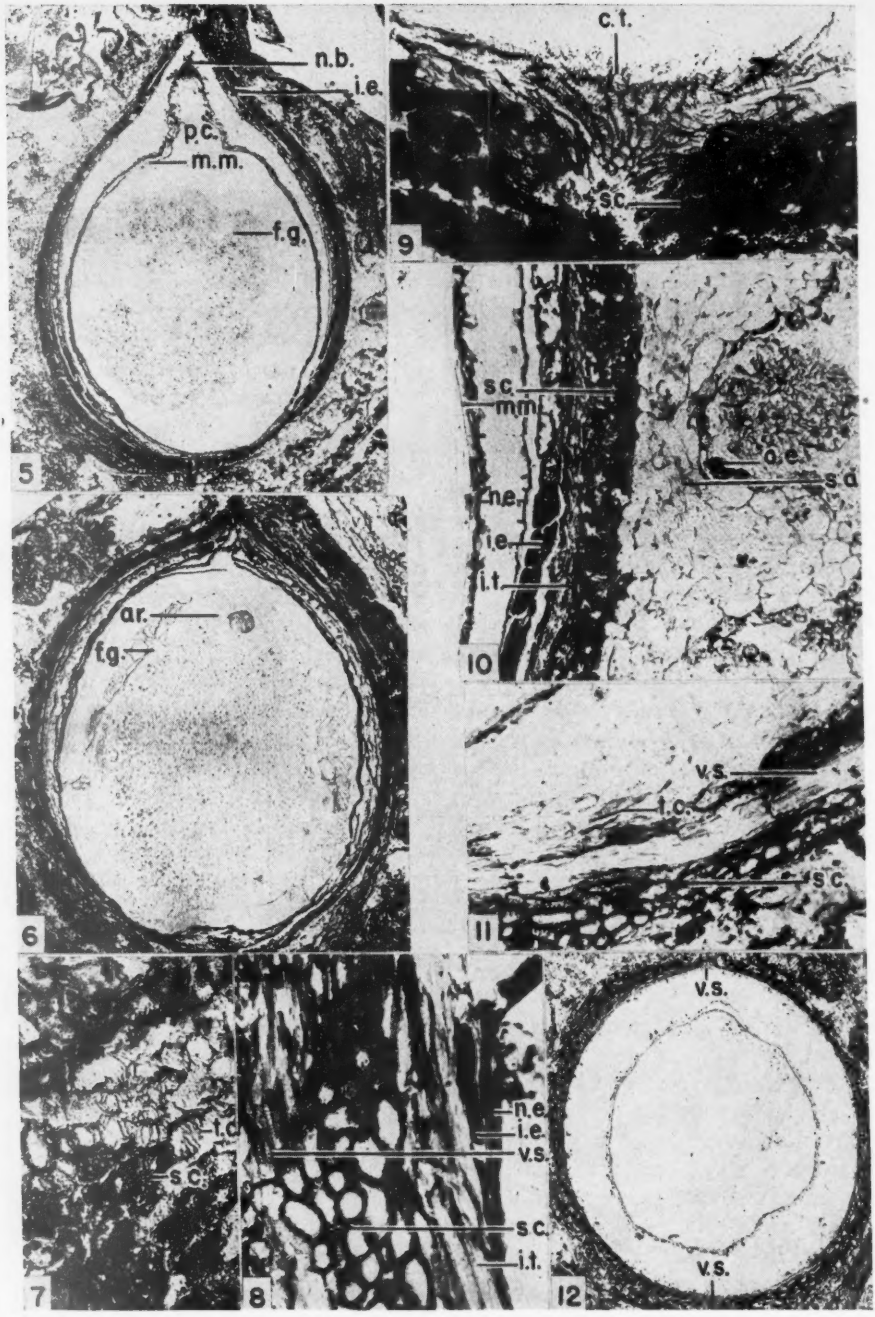
INTEGUMENT.—The undulating margin of the sarcotesta is delimited by a well-defined outer epidermis, in which the cell walls show evidence of thickening and cutinization (fig. 10). Inside the epidermis the sarcotesta is composed of larger parenchymatous cells, which are less well preserved. The thickness of this sarcotestal parenchyma zone varies from 0.1 to 0.4 mm., producing the uneven outline of the seed (figs. 3, 5, 10). The sarcotesta reaches its maximum expansion in the form of an irregular collar at the apex of the seed (figs. 3, 5).

A zone of sclerotesta, 0.06–0.08 mm. wide, adjoins the inner margin of the sarcotesta (fig. 10) and is composed of thickened cells in which circular pitting of the walls is occasionally preserved (figs. 7, 8). The cells of this zone are most frequently only slightly elongate in longitudinal section (fig. 8) and are of irregular form in transverse section (fig. 12). The sclerotestal band continues uniformly throughout the body of the seed, broadening only at the micropylar and chalazal regions (fig. 5). The basally expanded zone of sclerenchyma forms a broad, inverted cone, which is penetrated by the vascular strands, while its extension at the apex produces a short, micropylar beak (fig. 3).

A poorly preserved and less dense tissue, 0.02—0.04 mm. thick, persists inside the band of sclerotestal cells (figs. 8, 10).



FIGS. 1–4.—Figs. 1, 2, *Coronostoma quadrivasatum*, reconstructions of seed. Fig. 1, median longitudinal section. Fig. 2, transverse section at base of plinth. Fig. 3, *Taxospermum undulatum*, reconstruction of median longitudinal section. Fig. 4, *Conostoma quadratum*, reconstruction of median longitudinal section. *ar.*, archegonium; *f.g.*, female gametophyte; *i.e.*, inner epidermis of integument; *i.n.*, inner nucellar tissue; *int.*, integument; *i.t.*, soft, inner tissue of integument (endotesta); *la.*, lagenostome; *m.m.*, megaspore membrane; *n.b.*, nucellar beak; *n.e.*, nucellar epidermis; *o.e.*, outer epidermis of integument; *pa.*, palisade cells; *p.c.*, pollen chamber; *pl.*, plinth; *sa.*, sarcotesta; *sc.*, sclerotesta; *v.e.*, vertically elongate cells of integument; *v.s.*, vascular strand.



FIGS. 5–12.—*Taxospermum undulatum*, seed. Fig. 5, median section of type specimen, approx. ×14. Fig. 6, longitudinal section showing female gametophyte and an archegonium, approx. ×14. Fig. 7, portion of apex of longitudinal section, approx. ×193. Fig. 8, inner region of integument at level of fusion of nucellus with integument, approx. ×126. Fig. 9, base of seed, approx. ×97. Fig. 10, portion of type specimen showing sequence of tissues, approx. ×75. Fig. 11, vascular strand passing obliquely upward through sclerotesta in lower region of seed, approx. ×95. Fig. 12, transverse section in central region of seed, approx. ×14. *ar.*, archegonium; *c.t.*, chalazal tracheids; *f.g.*, female gametophyte; *i.e.*, inner epidermis of integument; *i.t.*, soft, inner tissue of integument; *m.m.*, megaspore membrane; *n.b.*, nucellar beak; *n.e.*, nucellar epidermis; *o.e.*, outer epidermis of integument; *p.c.*, pollen chamber; *sa.*, sarcotesta; *sc.*, sclerotesta; *t.c.*, transfusion cell; *v.s.*, vascular strand.

The inner margin of the integument is conspicuously bordered by an inner epidermis, in which the dark, thick-walled cells are larger than the cells of the outer epidermis or of the upper area of the nucellar epidermis (figs. 5, 10). The cells of the inner epidermis are even more enlarged in the lower flanks of the tapered apex of the seed, diminishing in size toward the micropyle (fig 5). In the lower area of the seed the inner epidermis disappears at the point of fusion of nucellus with integument (figs. 5, 8).

NUCELLUS AND FEMALE GAMETOPHYTE.—The nucellus is broadly fused to the integument for a distance of about one-tenth of the total height of the nucellus. The upper, free portion follows the inner contour of the integument up to the tapered apex. It then narrows into an elongate, conical pollen chamber, which is terminated immediately below the micropyle by a thickened nucellar beak (fig. 5). In the lower region of the free nucellus the epidermis is thickened into a form similar to that of the adjoining, inner epidermis of the integument (fig. 8). In preservation the nucellar epidermis is often folded, or the outer and inner walls of the cells may be torn apart (fig. 10).

Within the epidermis of the nucellus the heavily cutinized megaspore membrane, 9–11 μ in thickness, is intact, except for a broken arch in the pollen chamber (figs. 3, 5).

Sixteen specimens showed some preservation of the female gametophyte tissue. In these the gametophyte occupies from one-fourth to almost all the volume of the megaspore membrane; it is composed of organic material of varying density, but in most of the specimens there is little evidence of cellular structure (figs. 5, 6). In a few peel sections, areas appear to be composed of contracted cells, clinging together in a loose tissue. The gametophyte is raised at the apex into a low tent-pole (fig. 5), and on either side of this apical knob is a spherical to elliptical archegonium (fig. 6). The peripheral area of the venter of each archegonium is less dense than the surrounding gametophyte, while the central area is occupied by a single, dark, spherical body, which is considered to represent the egg or a zygote.

VASCULARIZATION.—A single vascular bundle enters the seed through the basal, inverted cone of sclerenchyma and continues unbranched to the broad, chalazal tracheal disk (fig. 9). From this level, two vascular strands diverge, pass obliquely outward through the sclerotesta (fig. 11), and curve upward at the outer margin of the sclerotestal ribs (fig. 8). Spiral to scalariform tracheids are preserved within the primary vascular bundle and the diverging strands. In addition, larger, proportionally shorter cells of transfusion tissue (fig. 11) are associated with the chalazal tracheal disk. Outside the sclerotesta, on either side of the micropyle, is a cushion of tissue which includes transfusion cells (fig. 7) and smaller, elongate, spirally thickened elements. The lateral vascular strands extend up to the level of the micropyle and then flare outward into the vascular cushions which are associated with the micropyle.

TAXONOMIC POSITION AND COMPARISON WITH OTHER SEEDS.—Although these seeds are small, as is characteristic of the Lagenostomales, this group was immediately removed from consideration because of differences in both the organization of the integument and the vascularization. The seeds differ from the radiospermic and the trigonal characters of the Trigonocarpales. In the presence of two outer vascular strands

the seeds correspond to the Cardiocarpales. The latter are usually strongly flattened laterally, and the base of the seed is commonly broadly rounded or slightly cordate. In these respects, however, the genus *Taxospermum* differs from other structurally preserved members of the Cardiocarpales—*Cardiocarpus*, *Cycadinocarpus*, *Rhabdospermum*, *Diplotesta*, *Mitrospermum*, and *Kamarospermum*. *Taxospermum* is only slightly flattened, with no pronounced lateral wings, and the shape of the seed is ovoid rather than cordiform.

In the course of the vascular strands in the seed, *Taxospermum* also differs from the other members of the Cardiocarpales. *Taxospermum* has no well-developed, vascular extensions from the chalazal tracheal disk in the nucellus, and the outer vascular strands which depart from the chalazal level are not recurrent. *Taxospermum* conforms to the general integumentary and vascular organization of the Cardiocarpales but is distinctive in the pattern of its vascularization.

The seed genera *Taxospermum* and *Sarcotaxus* were originally described by BRONGNIART (**6, 7**) from the deposits of St.-Étienne, which are of Stephanian age in the Upper Carboniferous. The slide preparations of BRONGNIART and of RENAULT were later restudied by BERTRAND (**5**), who emended the genus *Taxospermum* to include specimens which previously had been assigned to *Digonospermum* and *Sarcotaxus*. BERTRAND did not designate species among the specimens of the revised genus *Taxospermum*.

These new specimens of the American coal flora conform to the generic description of *Taxospermum* in the differentiation of the integument into sarcotesta and sclerotesta, in the presence of the conspicuous, inner epidermis of the integument, and in the course of the vascularization in the seed. The irregular sarcotesta and the nearly round transverse form of the sclerotesta of the new *Taxospermum* seeds would be most like *Sarcotaxus olivaeformis* and *S. angulosis*, as described by BRONGNIART (**6, 7**). The American seeds, however, are smaller and differ in the organization of the sarcotesta from the species which were described by BRONGNIART and which were later included by BERTRAND (**5**) in *Taxospermum*. The most conspicuous characteristic of the specimens from Illinois is the varying undulation of the periphery of the sarcotesta. This character is considered a useful one in distinguishing these new specimens of *Taxospermum*.

Taxospermum undulatum shows some similarity to *Leptotesta grand 'euryi*, whose anatomical structure was described by LOUBIÈRE (**23**). This small seed resembles *T. undulatum* in size and in the differentiation of the integument. The nucellus, as it was interpreted by LOUBIÈRE, however, is attached only at the summit. EMBERGER (**11**) has suggested that this condition of the nucellus is due to fossilization, and he would include *Leptotesta* among the seeds in which the nucellus is extensively fused with the integument, i.e., the Lagenostomales and the modern gymnosperms. If the seed *Leptotesta* with its contracted nucellus is to be reinterpreted, then this seed could just as logically be compared with those seeds with basal attachment of the nucellus, and *Leptotesta* would then be related to the Cardiocarpales and in that group close to *Taxospermum*. Further comparisons between *Leptotesta* and *Taxospermum* will depend upon the discovery of additional specimens of *Leptotesta*,

since the new genus and species were based upon a single specimen which had been found by GRAND 'EURY (**15**) and related to *Pecopteris pluckeneti*.

AFFINITIES.—Among the plant structures identified in the coal balls of the New Delta locality are cordaitean stems, cordaitean leaves, medullosan stems, and the medullosan-related *Alethopteris* leaves. Seeds of the Cardiocarpales are most frequently attributed to the Cordaitales, since winged, cordiform seeds, described under several genera of the Cardiocarpales, have been found attached to cordaitean axes. In more isolated cases, small, winged, lenticular seeds have been reported in attachment with pteridosperm foliage (**8, 15, 16, 32**). Seeds which have been found attached to medullosan foliage and stems have been of the Trigonocarpales type (**2, 8, 16, 19, 20**), so cordaitean affinities are more probable for *Taxospermum*. The possibility of a pteridosperm relationship for these seeds cannot yet be completely discounted, however. Until evidence appears showing attachment between these *Taxospermum* seeds and the associated cordaitean vegetative organs, no definite relationship can be established for the seeds.

DIAGNOSIS.—*Taxospermum undulatum* sp. nov. Ovoid seeds 4.5 mm. × 3.5 mm.; lateral flattening slight; two vascular strands depart obliquely upward from the chalazal tracheal disk, penetrate the sclerotesta, and rise at the outer margin of the sclerotestal ribs to the vascular cushions on either side of the micropyle; female gametophyte, when present, distally differentiated into a low tent-pole, flanked by two spherical to elliptical archegonia; periphery of the sarcotesta irregularly undulating.

Locality. New Delta, Williamson County, Illinois.

Horizon. Upper Carbondale group, Pennsylvanian system.

Type specimen. C.B.108d(2)III, Paleobotanical Collection of the Department of Botany, University of Illinois.

CORONOSTOMA QUADRIVASATUM GEN. ET SP. NOV.

GENERAL STRUCTURE.—Three specimens of this new seed have been discovered in coal balls from the upper part of the McLeansboro group of the Pennsylvanian system at the Berryville locality in Lawrence County, Illinois.

The elliptical seeds are 4.5 mm. in length × 3.25 mm. in maximum diameter (figs. 1, 13). In transverse section the apex is faintly lobed at the periphery. The lobing is more conspicuously developed at the inner margin of the integument, and alternate lobes of the canopy are correlated with the position of the four vascular strands (fig. 2). This apical lobing smooths to a round transverse section in the central region of the seed (fig. 16). There is no evidence in these specimens of the presence of a cupule.

INTEGUMENT.—At the outer margin of the integument a dense, fragmented line of epidermis follows the outline of the seed into the lining of the upper micropyle (figs. 1, 13). At the region of attachment and within the micropyle, the dark, epidermal band is evident as a layer of small, tabular cells.

Immediately within the epidermis the integument is differentiated into a uniseriate layer of radially elongate, palisade cells. This palisade zone widens near the apex of the seed and continues for a short distance into the lining of the micropyle (figs. 13, 14).

The inner area of the integument is occupied by a region of vertically elongate cells, which form a tissue several

FIGS. 13–20.—Figs. 13–16, *Coronostoma quadrivasatum*, seed. Fig. 13, median longitudinal section of type specimen, approx. ×12. Fig. 14, almost median section of area of lagenostome, approx. ×88. Fig. 15, base of seed showing chalazal tracheal disk and two departing vascular strands, approx. ×88. Fig. 16, slightly oblique transverse section in central region of seed, approx. ×14. Figs. 17–20, *Conostoma quadratum*, seed. Fig. 17, median section of apex, approx. ×95. Fig. 18, base of seed showing two of basal keels, approx. ×90. Fig. 19, slightly oblique longitudinal section which apically is closest to median, approx. ×14. Fig. 20, longitudinal section showing two of apical cusps of integument and portion of a basal keel, approx. ×14. *c.t.*, chalazal tracheids; *i.n.*, inner nucellar tissue; *la.*, lagenostome; *m.m.*, megaspore membrane; *pa.*, palisade cells; *pl.*, plinth; *v.e.*, vertically elongate cells of integument; *v.s.*, vascular strand.

cells in thickness. Except in the canopy, this vertically oriented tissue is of uniform width, ranging from 0.2 mm. to 0.3 mm. in thickness among the different specimens (figs. 13, 16). The integument is deeply furrowed on the inner face of the canopy (fig. 2), with the furrows flattening out above into the micropyle and below into the body of the seed. From the periphery inward the cells of the integument are increasingly enlarged and thin walled, and there is corresponding reduction in cell preservation. The soft inner tissue of the integument apparently was confluent with the nucellus up to the level of the plinth, but the preservation of this integument-nucellus boundary is fragmentary. In the canopy surrounding the lagenostome and the plinth the inner epidermis borders the poorly preserved, inner tissue zone of the integument, and in the lower canopy the outer wall of the inner epidermis is wrinkled and folded along the region in which the softer tissues are incompletely preserved (fig. 2).

NUCELLAR REGION.—The free epidermis of the upper nucellus arches inward and then rises abruptly into a cone, which is approximately 0.5 mm. in maximum width × 0.6 mm. in height (fig. 13). At the apex of this conical plinth the epidermis is further differentiated into a tubular lagenostome, which terminates below the outer micropylar opening (figs. 13, 14). The lagenostome tapers from a basal diameter of 0.17 mm. to 0.12 mm. at the apex. Its cells are smaller and more dense than those of the connected plinth zone below, and this provides the basis for delimiting the plinth and the lagenostome.

A linear residue of inner nucellar tissue curves below the plinth, and inside this layer occur a few traces of the megaspore membrane (fig. 13). In the region of the female gametophyte only a disorganized organic residue remains. The curved, apical pattern of the inner nucellar tissue and of the megaspore membrane suggests, however, that the female gametophyte may have been modified apically into a tent-pole.

Several elliptical microspores, averaging 40 × 50 μ, are present in the apex of the seeds, occurring in the plinth cavity, the lagenostome, and the upper micropyle. Some of these microspores have a marginal reticulate surface. None of the spores is intact enough, however, to permit comparison with illustrations of entire spores. Their ruptured condition suggests the possibility of a post-fertilization state.

VASCULARIZATION.—The single vascular bundle entering the seed continues upward into a narrow, chalazal tracheal disk from which arise four unbranched vascular strands (fig. 15). These pass upward in the soft tissue of the inner margin of the integument to the basal level of the plinth (fig. 1). There they extend into the expanded tissue zone of the canopy, tapering until they terminate near the micropyle (figs. 1, 13). Spirally thickened tracheids are the only cells which are preserved within the primary vascular axis, chalazal tracheal disk, and integumentary vascular strands. A transverse section in the central region of the seed shows the position of the four vascular strands in relation to the integument (fig. 16). Within a vascular strand the xylem occupies the inner portion of the bundle area, while the outer zone of the bundle, presumably occupied by the phloem, is not preserved. At the apex of the seed an extensive, unpreserved area accompanies the xylem tissue of each strand during its course in the canopy. This unpreserved tissue area may represent the former position of extra-vascu-

lar parenchyma, as well as phloem tissue.

COMPARISON WITH OTHER SEEDS.—Specimens of *Coronostoma quadrivasatum* exhibit the characteristics of the seeds assigned to the Lagenostomales, resembling the Lagenostomales in (*a*) the fusion of the nucellus with the integument, (*b*) the position of the vascular strands in the inner zone of the integument, and (*c*) the elaborate differentiation of a lagenostome at the apex of the nucellus. Also, the seeds of the Lagenostomales, like these new seeds, are typically small. GORDON (12) has summarized a comparison of the seed genera placed within the Lagenostomales. On the basis of such a comparison the seeds assigned to the new genus *Coronostoma* are seen to differ from the previously described genera of the Lagenostomales in a group of anatomical details which are related to the lagenostome.

Among the described genera of the Lagenostomales these new seeds most closely resemble *Conostoma* and *Lagenostoma*. Species of *Conostoma* have been described in both European and American coal-ball collections. In contrast, although species of *Lagenostoma* have been widely described in the European fossil flora, American reference to *Lagenostoma* has been made only in coal-flora enumerations by NOÉ (25) and DARRAH (9). Otherwise, comparisons have not been made between the European species of *Lagenostoma* and the reported American forms. Both *Conostoma* and *Lagenostoma* have almost complete fusion of the apical lobes of the integument around the lagenostome. The same fusion occurred in *Coronostoma*. Detailed anatomy of the integument of *Coronostoma*, including cell form and size, as well as tissue distribution, more nearly corresponds to that of *Lagenostoma*, as described by OLIVER and SCOTT (27) and by PRANKERD (28). *Lagenostoma* and *Coronostoma* differ in the form and position of the lagenostome. In *Lagenostoma* the cells of the plinth are scarcely differentiated from those of the flask-shaped lagenostome which extends through the outer micropylar opening. Within this lagenostome an extensive central column of nucellar tissue almost fills the pollen chamber. In contrast, the tubular lagenostome of *Coronostoma* is differentiated from the basally attached plinth region by a smaller size and a greater density of the cells. The lagenostome is inserted within the micropyle, where it terminates in the constricted upper micropyle below the opening. *Conostoma*, the other genus of the Lagenostomales which closely resembles *Coronostoma*, has a small lagenostome which is differentiated from the dome-shaped plinth and is inserted at the base of the micropyle. The new seed genus *Coronostoma* resembles *Lagenostoma* in the organization of the integument and is similar to *Conostoma* in the extent, but not the form, of lagenostome differentiation. The assemblage of lagenostome and plinth characters separates these new seed specimens from any previously described seed genus, and the genus *Coronostoma* is therefore established to include them.

AFFINITIES.—*C. quadrivasatum*, as one of the seed forms assigned to the Lagenostomales, is presumed to be the detached seed of a pteridosperm. The present evidence from attachment and association of the Lagenostomales suggests that *Coronostoma* would be related to stems similar in organization to *Heterangium* or *Lyginopteris*. The stem *Lyginopteris* (*Lyginodendron*) has only been mentioned from Illinois in the coal-flora enumeration of NOÉ (24), while *Heterangium* has been described in several localities (1, 14). *Heterangium* stems, as

well as medullosan foliage, petioles, and stems, occur in the coal balls of the Berryville locality. Seeds of the Trigonocarpales are usually attributed to the Medullosaceae, so *Pachytesta, Stephanospermum,* and *Rotodontiospermum*—the seeds of the Berryville coal flora which are classified as Trigonocarpales—are considered to be related to the associated medullosan vegetative organs. *Heterangium* is then more probable as a stem type which might have been related to *Coronostoma*. The stem *Heterangium grievii* has been assumed to be related to the seed *Sphaerostoma ovale* in the British coal flora (4). *Sphaerostoma* has not been reported in the American fossil flora. Among the American seeds of the Lagenostomales, several species of *Conostoma* also occur in association with *Heterangium americanum*. Both *Conostoma* spp. and *Coronostoma* are present in the Berryville coal flora, while *Conostoma platyspermum* and *C. quadratum*, but not *Coronostoma*, have been reported with *Heterangium americanum* from the Calhoun Mine of Richland County, Illinois. Species of *Conostoma* have also been found in association with *H. americanum* in the McLeansboro horizon at the St. Wendell's locality in Posey County, Indiana. It is possible that several seed genera of the Lagenostomales, including *Coronostoma quadrivasatum*, may have been related to stems of the type which are now assigned to *Heterangium*.

DIAGNOSIS.—*Coronostoma quadrivasatum* gen. et sp. nov. Elliptical seeds 4.5 mm. in length × 3.25 mm. in maximum diameter; slight external ridges of the fused apical lobes, while the lower seed is round in transverse section; four unbranched vascular strands ascend in the inner zone of the integument from a basal vascular bundle; nucellus and integument confluent up to the level of the plinth; conical plinth surmounted by a thickened, tubular lagenostome within the upper micropyle.

Locality. Berryville locality, Lawrence County, Illinois.

Horizon. Upper McLeansboro group, Pennsylvanian system.

Type specimen. C.B.15b(6). Paleobotanical Collection of the Department of Botany, University of Illinois.

CONOSTOMA QUADRATUM GRAHAM

A single, well-preserved specimen of this seed was found in coal-ball material collected in the Berryville locality of the Upper McLeansboro group in the Pennsylvanian system of Illinois. Since the coal ball was being peeled for another specimen, the peel series for this seed is complete from the outer margin of the integument past the median section.

GENERAL STRUCTURE.—The maximum diameter of the seed is 1.13 mm., and the maximum length of any of the sections is 3.9 mm. The elliptical seed is characterized by four large, apical projections and the same number of smaller, basal extensions of the integument (figs. 4, 18, 20). The section of the seed which shows the lagenostome in median section (fig. 19) does not include the identifying apical and basal structures of the integument, which appear in other peels from the same specimen. There is no evidence of the attachment of a cupule.

INTEGUMENT.—The outer line of the epidermal layer is preserved as an apparently detached, narrow band which follows the outline of the integument. In this specimen the epidermal layer is most evident at the base of the seed.

Within the epidermis is a uniseriate layer of cells which vary in form throughout the length of the seed (figs. 18, 20). In longitudinal section the cells appear

square to radially elongate on the basal extensions of the integument, vertically elongate upward in the body of the seed, and in the region of the micropyle again somewhat radially elongate.

The innermost preserved zone of the integument is a compact tissue of vertically elongate, thick-walled cells (figs. 18, 20). At the inner margin of this tissue there is only fragmentary preservation of a delicate, thin-walled region which extended to the nucellus.

Excluding the line of the epidermis, which was not in contact with the rest of the integument, the integument of the central region of the seed has an average thickness of 0.09 mm. It widens around the micropyle and terminates in four conspicuous cusps which encircle the micropyle (fig. 20), while the basally expanded integument extends into four small, flaring keels (fig. 18).

NUCELLAR REGION.—The nucellus was confluent with the soft, inner tissue of the integument to the level of the plinth. The nucellus and the inner epidermis of the integument then diverged, as the nucellar epidermis was differentiated into the plinth and the lagenostome (figs. 4, 19). The cells which form the dome-shaped plinth have a greater wall thickness than do those of the lower nucellar epidermis which was fused to the integument.

The sculptured, ellipsoidal lagenostome, 0.14 mm. in height × 0.23 mm. in width, is inserted in the depressed apex of the dome of the plinth, below the lower opening of the micropyle (fig. 17). Although cells of the lagenostome directly below the micropyle appear to be smaller and less rigidly organized than those on the flanks, no separated, upper opening was observed through half-a-dozen peel sections within the lagenostome. Several triradiate spores, approximately 16 μ in diameter, occur in the pollen chamber of the intact lagenostome. This spore size is not that of the microspore types which are usually considered to be gymnospermous, but, since the seed is intact, their presence within the pollen chamber is evidence that the lagenostome previously had been open. Its upper closure is then related to a stage of seed development.

In the lower seed the inner nucellar layer is represented by a distorted line of organic residue which is preserved inside the zone of confluent integument and nucellar epidermis (figs. 4, 19). At the apex the plinth cavity is delimited below by a continuation of the inner nucellar layer, which rises into an irregular mass of tissue, obstructing the lower opening of the lagenostome (fig. 17).

The cutinized megaspore membrane, 5–7 μ in thickness, follows, just inside it, the outline of the inner nucellus, ascending toward the lagenostome in a broken arch (figs. 4, 19). Although no female gametophyte is recognizable in this specimen, the elevated condition of the inner nucellar tissue and the apically broken megaspore membrane suggest a condition of maturity comparable with that of the gametophyte observed by LONG (**22**) in *Lagenostoma ovoides*.

VASCULARIZATION.—The single vascular bundle entering at the base of the seed is divided at the chalazal level into four vascular strands, which ascend unbranched in the soft, inner margin of the integument (figs. 4, 18). As the integument is thickened into the canopy surrounding the micropyle, the strands curve inward and penetrate the compact tissue of the canopy (figs. 4, 17). They do not enter the terminal cusps of the integument but, instead, terminate close to the micropylar opening. Within the vascular strands are found spiral to sca-

lariform tracheids and accompanying zones in which the tissue has not been preserved. Part of these areas may represent the former position of the phloem tissue.

COMPARISON WITH THE TYPE SPECIMEN OF CONOSTOMA QUADRATUM GRAHAM.—After comparison with the slides from the type specimen of *C. quadratum* Graham, this new specimen has been found to correspond so closely in several anatomical details that it has been assigned to the same species.

The original description of *C. quadratum* was based upon a series of transverse peels which were made from the lower portion of a single seed. It did not include the region of the lagenostome. From this incomplete transverse series, GRAHAM (**13**) gave *C. quadratum* the following diagnosis: "Seed squarish in cross section. Ribs absent. Radiospermic with four vascular bundles. Diameter 1.35 mm."

The specimen of *C. quadratum* described by GRAHAM and this new seed specimen were both preserved in coal balls from the McLeansboro group of the Pennsylvanian system of Illinois. The type specimen was collected at the Calhoun Mine in Richland County, while the new specimen was collected in the Berryville locality of the adjoining Lawrence County.

The two specimens have the same sequence of tissues in the integument: there is an outer, incompletely preserved epidermis, a uniseriate layer which may show palisade form, a compact integument zone, and an inner, soft tissue, in which four unbranched strands ascend from a single, basal vascular bundle. The lowest sections of the type specimen show increasing angularity, and the uniseriate palisade tissue occupies more of the transverse area. This organization corresponds closely with the longitudinal sections of the flaring basal keels in the new specimen of *Conostoma*. The difference between the maximum diameter of the new specimen and that of the type specimen results from the inclusion of the fragmentary epidermal layer in the measurement of the transverse sections of the type specimen.

The new specimen agrees closely with *C. quadratum* in size and in the structural details which can be determined from the limited number of transverse sections of the type specimen. This new seed is therefore considered to be a specimen of *C. quadratum* and thus to furnish additional, descriptive information concerning the area of the lagenostome and the general aspect of the longitudinal section of the seed.

COMPARISON WITH OTHER SEEDS.—GRAHAM (**13**) observed that *C. quadratum* more closely resembled the structure of *C. anglo-germanicum* Oliver and Salisbury (**26**) than that of *C. oblongum* Williamson (**33**). The sections of the new specimen re-emphasize this similarity. *C. anglo-germanicum*, occurring in the Lower Coal Measures of England and Germany, is a larger, cylindrical seed, measuring 7 mm. × 2.3 mm., and is characteristically eight ribbed, with the four major ribs extending into apical projections. The basal differentiation of the ribs of *C. anglo-germanicum* is, however, not so sharply defined as the basal keels of the new specimen of *C. quadratum*. The basal origin and the inner course of the four vascular strands are the same in *C. quadratum* and *C. anglo-germanicum*, and the two species have a similar distribution of tissues within the integument. The details of this new specimen further support *C. quadratum* as a species which is structurally close to *C. anglo-germanicum*.

Among American *Conostoma* material which has been reported, the specimen of *C. oblongum* described by KERN and ANDREWS (18) from the Des Moines series of the Pennsylvanian system of Iowa varies from the descriptions and illustrations of European specimens of *C. oblongum*. These points of variance are points of resemblance to *C. quadratum* and *C. anglo-germanicum*. Since the first section of the Iowa specimen was beyond the median plane, the complete details of vascularization and of apical and basal modification are not available. The Iowa specimen of *C. oblongum* in its cylindrical form, in its apical lobing, and in the appearance of the lagenostome does suggest characteristics of *C. quadratum*.

AFFINITIES.—The genus *Conostoma* is placed in the Lagenostomales, a seed group of pteridosperm affinity. Among the British Lagenostomales, the seed *Lagenostoma lomaxi* has been related to the stem *Lyginopteris oldhamia*, and *Sphaerostoma ovale* is considered as the probable seed of *Heterangium grievii*. In contrast, no American or European species of *Conostoma* has yet been definitely related to a particular stem or foliage genus. Stems of *Heterangium americanum* have been found in association with *Conostoma quadratum* in the McLeansboro horizon of both Richland and Lawrence counties. Other species of *Conostoma* and at least one other genus of the Lagenostomales, *Coronostoma*, however, are also present in these coal balls, so the association of *C. quadratum* and *Heterangium* is not conclusive, but it is of descriptive interest.

Summary

1. An anatomical study has been made of additional, small petrified seeds from two Illinois coal-ball localities.

2. *Taxospermum undulatum* sp. nov., an ovoid seed with only slight lateral flattening, is included in the Cardiocarpales.

3. *Coronostoma quadrivasatum* gen. et sp. nov. has structural characteristics which relate it to the seed genera *Conostoma* and *Lagenostoma* in the Lagenostomales.

4. *Conostoma quadratum* Graham, which was originally described only from transverse sections, is more completely described from longitudinal sections which include the region of the lagenostome and the apical and basal modifications of the integument.

It is a pleasure to acknowledge the guidance of Professor WILSON N. STEWART, University of Illinois, under whose supervision this research was conducted. Professor G. NEVILLE JONES, University of Illinois, offered suggestions on nomenclature, and E. DERROUGH KIRK, University of Illinois, assisted in the preparation of the illustrations.

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EFFECT OF BORON ON GERMINATION OF FLAX SEED AND ON RESPIRATION OF FLAX SEEDLINGS

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY **628**

WILLIAM JENSEN

Introduction

Knowledge of the use of boron by plants is limited, and the element has been considered to play various roles at different times. Previous work has shown that it significantly affects actively metabolic tissues. Since in germination the entire plant is in a highly active metabolic state, this is a logical point of investigation. To date, however, little of such study has been done.

HECKEL in 1875 and ARCHANGELI in 1885, whose work was reviewed by BRENCHLEY (**1**), allowed various seeds, including flax, to germinate in rather high concentrations of H_3BO_3 and observed only inhibition. COOK (**2**) in 1916 did work with boron toxicity, where again high concentrations were used and only inhibition of germination was observed.

In the present work much lower concentrations of boric acid and of two sodium borates were used. Measurements were made of (*a*) the rate of germination and (*b*) the rate of respiration (as measured by O_2 uptake) of the germinating seeds.

Material and methods

Flax seed (*Linum usitatissimum*) was used in all experiments because it is a small, highly viable, rapidly germinating seed that is easy to obtain and handle. The reagents were boric acid (H_3BO_3), sodium metaborate ($NaBO_2$), sodium tetraborate ($Na_2B_4O_7$), manganese sulfate ($MnSO_4$), and zinc sulfate ($ZnSO_4$), all of C.P. grade. Water distilled in a copper still was used in preference to water redistilled in Pyrex, in an effort to keep the boron concentration in the water as low as possible. A supply of water with a relatively constant amount of boron in it was obtained by filling a large soft-glass carboy with distilled water and using only this for solutions.

Soft glass was used wherever possible to keep to a minimum the amount of boron coming into solution. All solutions were kept in soft-glass bottles, and soft-glass Petri dishes were used for germination chambers.

All the reagents were prepared in molar concentration and diluted to the following concentrations: $10^{-2}\,M$, $10^{-4}\,M$, $10^{-6}\,M$, $10^{-8}\,M$, $10^{-10}\,M$.

The standard procedure was to place twenty-five seeds on a single sheet of filter paper on the bottom of a Petri dish and then add 2.5 ml. of the solution to be used. The dishes were then placed in the dark in a constant-temperature box at 28° C.

In the germination experiments a group of four replicates of twenty-five seeds each was used for each different treatment. Normally, the five treatments and a control in distilled water were run at the same time. A seed was considered as germinated after the root had emerged from the seed coat. Since the root is white and the seed coat brown, the time of emergence could be deter-

mined accurately. One difficulty presented itself when some of the roots appeared unable to rupture the seed coat and pushed the cotyledons out through the other end of the seed. This appeared to be a normal phenomenon and usually occurred in less than 5% of the seeds in all controls and treatments. A few seeds usually germinated after 12 hours. Counts were started at 15 hours and made at 2- or 4-hour intervals until the twenty-fifth hour, at which time usually more than 75% of the seeds in the control had germinated. Total germination varied from 85 to 95%.

In order to make the counts, seeds were taken from the dark and examined in the light. It was therefore necessary to know whether the light used in making the early counts had an effect on the results obtained at later countings. To determine this, an experiment was performed in which a number of samples were started at the same time, and at each time interval a different sample was counted. Thus each count represented a sample that had not been exposed to light before counting. There was no significant difference between results obtained by this method and those obtained by repeated countings in light. Therefore, in all subsequent germination experiments, light was employed at the time of counting.

In the respiration experiments the same basic germination technique was employed. Six groups of twenty-five seeds each were germinated for each treatment. Respiration was measured with a standard Warburg manometer. Seventy-five seeds (three combined groups) were placed in a vessel with 2.0 ml. of fresh solution with the same boron concentration as that which the seeds had been in previously. To absorb the CO_2, 0.2 ml. of NaOH was placed in the center well. Two vessels were used for each concentration and the control. The bath temperature was 28° C. Readings were taken at 15-minute intervals, and each run was 75 minutes long.

No buffer was used because the mucilage exuded by the seeds seemed to act as a natural buffer over a short range near pH 6.5–7.0, approximately the pH values of the solutions used, except for the highest concentration. To insure that the results were not affected by leaving the solutions unbuffered, several respiration and germination runs were made with 0.01*M* phosphate buffer at pH 6.5 or 7.0. There was no essential change in pattern of reaction from that of unbuffered material.

The advantages in using seeds as test organisms lie in the facts that (*a*) the effects of one-salt solutions can be studied without interference of growth because of lack of other salts; (*b*) experiments can be completed in shorter lengths of time than with more mature plants; (*c*) larger samples can be employed; and (*d*) in respiration runs, numerous whole, uninjured plants can be used. The disadvantages include (*a*) the large natural variation of the seeds; (*b*) the lack of data on whether a given seed produces germination inhibitors and how these may affect respiration; and (*c*) the changing of the seed with aging.

Results

EFFECT OF BORON ON GERMINATION.—Figure 1*A* is a summation of data on the effect of boron on germination. Since results for H_3BO_3, $NaBO_2$, and $Na_2B_4O_7$ were for all purposes identical, only data for H_3BO_3 are shown. This graph represents results of six trials with four replications at each point. Data for four experiments with four replications for each salt are not shown. At a concentration of

10^{-6} *M* there was a definite stimulation of germination and some evidence of stimulation at 10^{-8} *M*, while it appears that there was none at 10^{-4} *M* and at

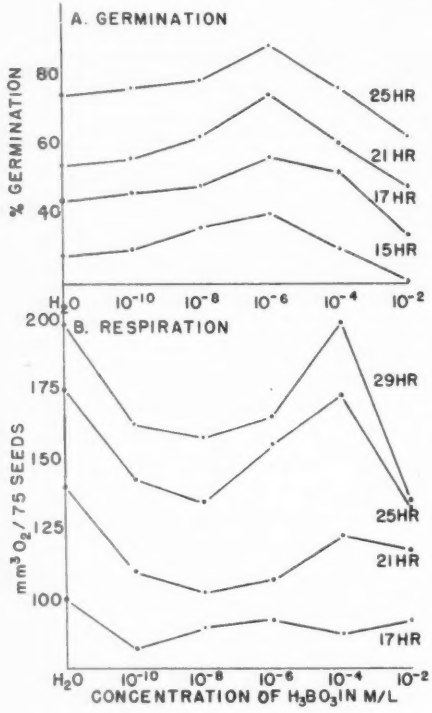

FIG. 1.—Effect of H_3BO_3 on germination (*A*) and respiration (*B*) of germinating flax seed at various time intervals after moistening seeds.

10^{-10} *M*. At 10^{-2} *M* apparently boron somewhat inhibited germination. Not included in the graph are data for boric acid at molar concentration which was, for all purposes, completely inhibitory.

EFFECT OF MANGANESE AND ZINC ON GERMINATION.—Germination experiments were run with $MnSO_4$ and $ZnSO_4$ to determine whether other recognized trace elements produced the same effect as boron. Figure 2 shows that they did not. These salts stimulated germination with increase in concentration. Little more was done with these salts, but the difference in pattern is interesting and may, in future work, prove important.

EFFECT OF BORON ON RESPIRATION.—Figure 1*B* is a summation of data concerning the effect of boron on the respiration of germinating flax seed. Here again results obtained for H_3BO_3, $NaBO_2$, and $Na_2B_4O_7$ were so similar that only data for H_3BO_3 are shown. This graph represents four experiments, with each point having two replicates per experiment. For each salt three experiments were run with two replicates at each point, as with the acid.

The pattern is quite clear for all the time intervals above 17 hours. There was a marked depression at 10^{-10} *M* and 10^{-8} *M*, a slightly smaller depression at

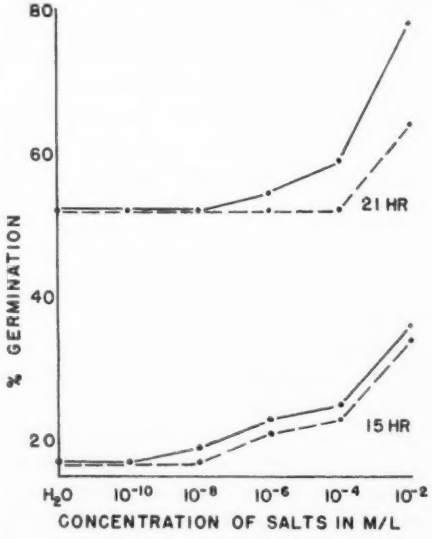

FIG. 2.—Effect of $MnSO_4$ (*solid line*) and $ZnSO_4$ (*broken line*) on rate of germination of flax seed at various time intervals after moistening seeds.

10^{-6} *M*, no depression at 10^{-4} *M*, and an increasingly greater depression at 10^{-2} *M* with elapsed time. Figure 3 represents the respiration of seeds in water, in

10^{-10} *M* H_3BO_3, and in 10^{-2} *M* H_3BO_3, as a function of time rather than concentration. The difference in respiration between seeds in water and those in 10^{-10} *M* H_3BO_3 remained essentially constant throughout the experiment. The difference between the control and the seeds treated with 10^{-2} *M* H_3BO_3 became increasingly greater from the start to the end of the experiment. This observation is important when the question arises as

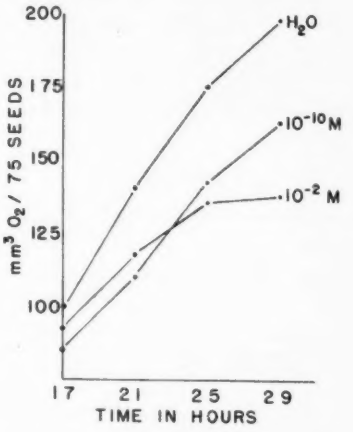

FIG. 3.—Effect of various concentrations of H_3BO_3 on respiration of flax seedlings.

to what affected the respiration and what affected the germination. The germination rates of the seeds in water and in 10^{-10} *M* H_3BO_3 were essentially the same, while the germination of the seeds at 10^{-2} *M* was not so rapid as that of the control.

When figure 1*A* and *B* are compared, little correlation between the shapes of the two sets of curves is evident, except at 10^{-2} *M* and 10^{-4} *M*. These are the only concentrations at which germination and respiration seemed directly correlated—in 10^{-2} *M* they were both lower and in 10^{-4} *M* they were both approximately the same as in water. The maximum stimulation of germination by boron occurred at 10^{-6} *M*, while the maximum inhibition of respiration was at 10^{-8} *M*.

Discussion

Reference was made in the introduction to early investigators who worked with H_3BO_3 and the germination of seeds. HECKEL and ARCHANGELI used flax seed and boron concentrations ranging from 0.01 to 1% H_3BO_3—roughly equivalent to between 10^{-3} *M* and 10^{-1} *M;* they observed either little effect or growth retardation. Results in the present work confirm this.

The pattern of germination, where the rate was directly correlated with the concentration of boron until a toxic concentration was reached and then inversely correlated with concentration, is paralleled by the work done with more mature plants by numerous workers (**1, 3, 5, 6, 8**). WARINGTON (**8**) and MORRIS (**5**) observed a difference between the effects of acid and sodium metaborate directly related to the latter's having four boron atoms per molecule while the former has but one. No such difference was observed in these experiments, but this was undoubtedly the result of large dilutions, low concentrations, and the fact that the growth measurement used here —rate of germination—was not so critical as theirs. MORRIS measured root growth of seedlings, while WARINGTON used total fresh and dry weights of broad bean and barley.

A significant point should be mentioned relative to the action of boron acids and their salts in aqueous solutions. Inorganic chemists (**7**) apparently are of the opinion that, regardless of the acid or salt used, once it is placed in an aqueous solution, it forms a number of ionic species having but one boron atom each. These species are in equilibrium with one

another at a given concentration, but this equilibrium changes with the concentration in respect to both number of ionic species and percentage of ions of each species. It must be emphasized that this is not a simple case of an acid dissociation but a complex phenomenon based only partially on this and partially on certain solubility reactions. Simplifying the problem, it may be said that, at greater concentrations of both the acid and the salts, the higher species, which may be represented for convenience as the undissociated H_3BO_3, are present in predominance, while at lesser concentrations the lower species, which may be represented as $H_2BO_3^-$, are present in predominance.

Now, if $H_2BO_3^-$ inhibits respiration and H_3BO_3 does not affect respiration, then one may expect an effect on respiration at lower concentrations and none at the higher concentrations. This is what the respiration data indicate. An exception is 10^{-2} *M;* this can be clarified by explaining the germination data.

From data presented here and by other workers it appears that the effect of boron on growth is directly correlated with the total amount of boron present. Thus the stimulatory effect on germination is positively correlated with concentration until the amount becomes toxic. The lowered respiration at 10^{-2} *M* is probably due to this toxic effect of the total boron present at high concentration and not due to the $H_2BO_3^-$ ion, as at lower concentrations. This is clearly indicated in figure 3.

It is suggested, then, that the effect of boron on respiration is a function of the amount of $H_2BO_3^-$ present, while the effect on germination rate is a function of the total boron present. Furthermore, these functions appear to be independent of each other, particularly at 10^{-6} *M* where there was an inverse relation between respiration and germination.

This conclusion, while interesting, in that it indicates two functions for the boron atom, does not provide an answer as to how boron affects the germination rate or the respiration rate. It should be remembered that flax is a fatty seed with much food material in the form of oils. MARSH and SHIVE (4) showed that in the corn plant there was a definite relation of boron to amount of free calcium and to fat metabolism. The pronounced effect of the $H_2BO_3^-$ ion on respiration in the flax seed may be related to fat metabolism. Further work may clarify this problem.

Summary

1. Flax seed was germinated in various concentrations of H_3BO_3, $NaBO_2$, or $Na_2B_4O_7$. The rate of germination was high in comparison with the control in water in those seeds placed in concentrations of 10^{-8} *M* and 10^{-6} *M*. Above these concentrations the rate was lower; below these there was no difference in rate when compared with the controls.

2. Flax seed was also germinated in various concentrations of $MnSO_4$ and $ZnSO_4$. The rate of germination was higher in comparison with the control in those seeds placed in concentrations of 10^{-6} *M* and above and was directly correlated in rate with the higher concentrations.

3. The respiration of flax seeds germinating in the boron solutions was measured. There was a constant depression of respiration at 10^{-10} *M*, 10^{-8} *M*, and 10^{-6} *M*, no depression at 10^{-4} *M*, and an increasingly greater depression of respiration at 10^{-2} *M*, in comparison with the control.

4. A hypothesis is presented in which the effect of boron on the respiration of the germinating flax seed is explained as

a function of the $H_2BO_3^-$ ion, while the effect on germination is explained as a function of the total boron present. The lowered respiration at 10^{-2} *M* is the only case in which respiration seems affected by total boron—in this case the action is toxic.

The writer wishes to express appreciation to Dr. L. BOGORAD for guidance and suggestions throughout the course of this investigation.

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STUDIES ON THE METABOLISM OF PLANT NEOPLASMS
I. OXYGEN UPTAKE OF TOMATO CROWN-GALL TISSUES[1]

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 629

GEORGE K. K. LINK AND DAVID R. GODDARD[2]

Introduction

This paper will present two sets of findings pertaining to the oxygen uptake of crown gall of tomato. The first set is the result of a comparative manometric time-course study, from the seedling stage until flowering, of the oxygen uptake of the hypocotyl of the normal tomato plant and of the same organ inoculated with *Agrobacterium tumefaciens*, the incitant of crown gall of plants. The second set of findings includes the results of (*a*) a comparative manometric determination of the oxygen uptake of the normal and inoculated hypocotyl of tomato plants grown at two levels of nitrate-nitrogen nutrition, and (*b*) determination of the total nitrogen content of these organs sampled at the time of the manometric tests.

[1] This research was supported in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago. We are indebted to the Fels Foundation for use of space and equipment in the Herbert Jones Chemical Laboratory of the University of Chicago.

[2] Fellow of the John Senior Guggenheim Foundation, 1942–43.

Material and methods

All plants came from a single lot of certified tomato seed (*Lycopersicon esculentum* Mill., var. Bonny Best) and were grown in quartz sand in 6-inch pots. The sowing for the plants of the time-course

study was made on May 9, 1943. Beginning May 20, each pot received weekly an application of a complete mineral nutrient solution (Shive's R_2S_5). On May 28 the plants were thinned to five per pot. On June 1 half of the plants were inoculated in the middle of the fully elongated hypocotyl by puncture with a trident needle dipped into a culture of *A. tumefaciens* on agar (University of Chicago strain S 5–6). The control plants were punctured with a sterile trident. There was a slight development of wound tissue in the controls for about a week. In the inoculated plants gross tumor formation was evident within 5 days, and tumor development continued until the close of the study, when the tumors had begun to crack and show superficial and internal necrosis.

The sowing for plants of the nutritional tests was made August 30, 1943. From the outset, half of these plants were given the same nutrient solution as those of the summer series, while the other half received a Shive's nutrient solution in which calcium nitrate was replaced by calcium chloride. The plants receiving nitrogen were designated +N, the others, −N. After thinning on September 19, half of each lot of +N and −N plants were inoculated on September 21 in the middle of the fully elongated hypocotyl as in the spring tests; the other half, serving as controls, were punctured with a sterile trident. The behavior of control and inoculated plants as to wound response and tumor formation was essentially the same as that of plants of the spring sowing.

All manometric tests were made with standard Warburg vessels at 25° C. A buffer solution, as follows, was used: potassium phosphates, 0.01 M; $CaCl_2$, 0.005 M; $MgSO_4$, 0.002 M; pH 6.0. Three milliliters of this buffer and the tissues were placed in the main compartment of the vessel, and 0.3 ml. of 15% potassium hydroxide was added to the center cup. Readings were made at 20-minute intervals for 2–4 hours. In preliminary tests the young and very succulent hypocotyls were bisected transversely and/or longitudinally to determine whether slicing without subsequent washing had any appreciable effect on the rate of their gas exchange. It did not. Accordingly, in subsequent tests the control hypocotyls and the tumors were sectioned transversely into thin slices which were weighed and then placed without washing into the buffer solution in the vessels. Later, when the tumors had become quite large, they were quartered before slicing.

Preliminary tests also showed that the rate of oxygen uptake of tissues from the upper, middle, and lower thirds of the normal hypocotyl was the same. Hence, the slices were cut mainly from the middle and, to some extent, from the upper and lower thirds of the hypocotyls. The slices designated "tumor" in figures 1 and 2 were cut only from the sites of inoculation and, after the tumors were grossly recognizable, from the tumor tissues. Those designated "tumor ends" were made from the tissues contiguous to the tumor. They did not show any gross evidence of change at the time of cutting. However, as the tumor progressively involved the upper and lower third of the hypocotyl, the "tumor end" slices necessarily were obtained progressively higher up from the upper third, and lower down from the lower third of the hypocotyl.

The first test in the time-course study was made on May 31 with intact hypocotyls which had attained their mature length and had an average fresh weight

of 52.8 mg. On June 2 (a day after inoculation) the first test was made with punctured control and inoculated hypocotyls. Thereafter, manometric runs were made at various intervals up to July 22 (52 days after inoculation), when both control and tumor-bearing plants had begun to flower. The results of these tests are expressed as cu mm O_2/gm fresh wt/hr (fig. 1). Each point on the curves is the average of readings from two vessels as it also is in figure 2.

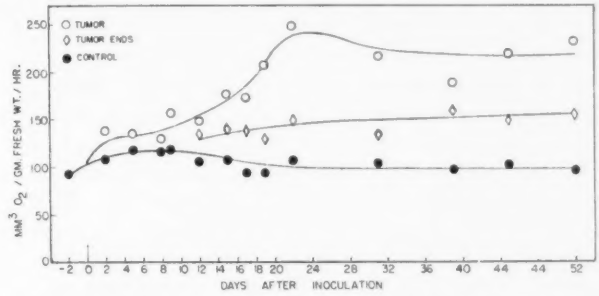

FIG. 1.—Changes in rates of oxygen uptake of slices of control and tumorous hypocotyl tissues during tumor development. Each point on curves represents average of readings from two vessels.

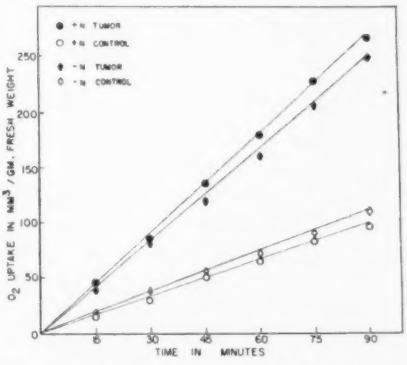

FIG. 2.—Oxygen uptake, 22 days after inoculation, of slices of control and tumorous tomato hypocotyl tissues grown with +N and −N.

On September 20, 1943, a comparative manometric study of oxygen uptake of control and inoculated hypocotyls of +N and −N plants was begun. Unfortunately, light-intensity conditions became so poor after mid-October that the −N plants began to die at their tips. The plan to parallel the tests of the spring series with tests of +N and −N plants until flowering time consequently had to be abandoned. Fortunately, on October 12, 22 days after inoculation, a complete set of samples for lyophilization and subsequent total-nitrogen determinations was collected, and manometric determinations of oxygen uptakes of both control and tumorous hypocotyls of the +N and −N plants were made. At this time light conditions still were adequate, and the −N plants had not yet begun to die. They had ceased to grow, but their tumors still were enlarging at the expense of the rest of the plant. The results of the manometric tests of October 12 are expressed as cu mm O_2/gm fresh wt/hr (fig. 2). Although they apply to only one stage in the development of this lot of plants, they are representative of the kind of differences noted in the other tests of the +N and −N plants. Results of nitrogen determinations of the lyophilized samples by the semimicro Kjeldahl method are given in table 1. We are indebted to Dr. MIYOSHI IKAWA for the analyses which were carried out in the

laboratory of Professor KARL P. LINK of the Department of Agricultural Chemistry of the University of Wisconsin. The values given in table 1 represent averages of quadruple analyses.

Results

Figure 1 shows that the rates of oxygen uptake of slices of the tissues of the control hypocotyl rose slightly from the beginning of the tests until about the fourteenth day. After that they decreased again, leveling off at about the initial rate. The rates of oxygen uptake of slices of the inoculated portion of the hypocotyl and, later, of the tumor were higher than those of the normal hypocotyl on the first day after inoculation and continued so until the close of the tests. The rates rose rapidly from the twelfth to the twenty-third day after inoculation and then fell off slightly. The rates of oxygen uptake of slices of the tumor ends were considerably higher than those of control hypocotyls and rose slightly throughout the period of the tests.

TABLE 1

PERCENTAGE TOTAL NITROGEN ON DRY-WEIGHT BASIS OF CONTROL AND INOCULATED TOMATO HYPOCOTYLS UNDER PLUS- AND MINUS-NITROGEN NUTRITION 22 DAYS AFTER INOCULATION

HYPOCOTYL SEGMENT	+N NUTRITION		−N NUTRITION	
	Control	Inoc.	Control	Inoc.
Upper one-third	4.25	4.37	0.95	1.48
Middle one-third	4.03	4.68*	0.84	2.09*
Lower one-third	4.09	3.82	0.87	1.13

* Tumor tissue only.

Figure 2 shows that 22 days after inoculation the rates of oxygen uptake of slices of tumors of the hypocotyls of both +N and −N plants were greater than those of the corresponding tissues of control hypocotyls and that the differences between the rates of slices of tumors of +N and −N plants were slight.

Table 1 shows that the accumulation of total nitrogen in tumorous tissue (calculated as a percentage of the dry weight) compared with its accumulation in the control hypocotyls was quite marked when nitrogen was available only in limiting amounts, but slight when the nitrogen supply was fairly adequate. In spite of this accumulation, the tumorous tissues from hypocotyls grown without exogenous nitrogen additions still contained a considerably smaller percentage total nitrogen than the comparable tumorous tissue from +N plants. The difference in nitrogen composition of the two sets of control hypocotyl tissues was even more marked.

Discussion and conclusions

The results show that, when calculated on a basis of fresh weight, the rates of oxygen uptake of slices of crown-gall tissues of the hypocotyl of tomato were at all times greater than those of slices of noninoculated hypocotyls, indicating a difference in kind and/or rate of metabolism. The differences in rate of oxygen uptake of slices of control and inoculated hypocotyls at different stages of development in the life-histories of the plants indicate that the rates of oxygen uptake by both control and tumorous tissues, especially of the latter, are in part functions of the stage of development of the tissues at time of slicing. Data bearing on the rate of gas exchange of tumorous tissue without specification of the stage of its development or of its age consequently must be used with great caution. The results of

the time-course study reported here indicate that there may not be one characteristic rate of oxygen uptake of crown-gall tissues. Possibly normal and abnormal plant tissues have fewer and/or less effective regulatory mechanisms than animal tissues. Results from manometric tests of tissues of one stage of development of the control plant and of the tumor apparently cannot, without qualification, be applied to another stage of development in comparative studies of the metabolism and of histo- and organogenesis of the tumor.

The results also indicate that in comparative studies of the oxygen uptake of crown-gall and normal tissues it is not permissible to use the apparently nonaffected parts of the tumorous organ as a control. For adequate controls in such studies tissues must be obtained from the same organ of a noninoculated plant of the same genetic constitution with the same past history (excepting inoculation) as to environment, nutrition, etc., and of the same age, and in the same stage of development. The finding that the rate of oxygen uptake of the tumor ends is higher than that of the tissues of the noninoculated organs indicates that metabolic changes in the later stages of tumor development are not dependent upon the presence of bacteria which initiate tumor development.

In spite of quite marked differences in the total nitrogen percentage of tumorous and control hypocotyl tissues grown with adequate exogenous supplies of nitrogen compared with the nitrogen composition of equivalent tissues grown under −N conditions, these differences are not reflected in the rate of oxygen uptake calculated on a fresh-weight base. It would appear that the oxidative capacities of control and tumorous hypocotyl tissues of the tomato are regulated by mechanisms peculiar to the cells of the tissues under consideration and are influenced slightly, if at all, by the nutritional status of the plant relative to nitrogen. It can only be inferred that real differences in the metabolic activities of normal and tumorous cells must be based on some constant character of the particular cells. Tentatively, it would not appear that total nitrogen is that character.

Although these findings show that normal and crown-gall tissues of the tomato hypocotyl differ in their rates of oxygen uptake, they differ from the generally accepted findings relative to normal and cancerous animal tissues. In the latter the rate of the oxidative phase of respiration is less than in normal tissues, while the rate of the obligate anaerobic phase is greatly enhanced over that of normal tissues. WHITE (2) has reported that on a dry-weight basis crown-gall tissues of the sunflower (*Helianthus annuus*) respire at a lower rate than the normal tissues. His findings for sunflower accordingly are at complete variance with the findings reported here for the tomato axis. WHITE also raised the point as to whether data calculated on the basis of protein-nitrogen instead of dry-weight determinations would have led to different conclusions. This and other pertinent matters have been under investigation in the Chicago laboratory by KLEIN (1).

Summary

1. A comparative manometric time-course study was made, from the seedling stage until flowering, of the oxygen uptake of the hypocotyl of the normal tomato plant (*Lycopersicon esculentum*) and of the same organ inoculated with *Agrobacterium tumefaciens*.

2. The oxygen uptake of the normal and inoculated hypocotyl of tomato plants grown at two levels of nitrate-nitrogen nutrition also was measured manometrically, and the total nitrogen contents of these organs, sampled at the time of one of the manometric tests, were determined.

3. Calculated on a basis of fresh weight, the rates of oxygen uptake of slices of crown-gall tissues were at all times greater than those of slices of the control tissues.

4. The rates of oxygen uptake by both control and tumorous tissues, especially of the latter, appear to be in part functions of the stage of development of the tissues at time of slicing.

5. It is indicated that in comparative studies of the oxygen uptake of crown-gall and normal tissues it is not permissible to use the apparently non-affected parts of the tumorous organ as a control.

6. Differences in the total nitrogen percentage of tumorous and control hypocotyl tissues grown with adequate exogenous supplies of nitrogen compared with the nitrogen content of equivalent tissues grown under −N conditions are not reflected in the rate of oxygen uptake calculated on a fresh-weight base.

7. Total nitrogen does not appear to be a more adequate basis than fresh weight for calculating the rates of oxygen uptake of crown-gall and control tissues.

DEPARTMENTS OF BOTANY
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STUDIES ON THE METABOLISM OF PLANT NEOPLASMS. II. THE TERMINAL OXIDASE PATTERNS OF CROWN-GALL AND AUXIN TUMORS OF TOMATO[1]

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 630

GEORGE K. K. LINK AND RICHARD M. KLEIN

Introduction

This paper presents the results of a study to determine (*a*) whether heavy-metal enzymes mediate oxygen uptake of normal and pathic tissues of the hypocotyl and internodes of the tomato (*Lycopersicon esculentum* Mill. var. Bonny Best) and (*b*) the percentages of this uptake mediated by iron and copper enzymes.

[1] These studies were supported in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago. We are indebted to the Fels Foundation for use of space and equipment in the Herbert Jones Chemical Laboratory of the University of Chicago and to Professor HANS GAFFRON for aid in the planning and execution of the experiments.

Material and methods

All plants for the initial tests came from a sowing made on August 30, 1943, which also supplied some of the plants

used for the study reported in the companion paper (**6**).

From the outset these plants were grown in 6-inch pots in pure quartz sand and received weekly an application of a complete mineral nutrient solution (Shive's R_2S_5). After thinning to six plants per pot on September 19, control plants were each punctured in the middle of the fully elongated hypocotyl with a sterile trident needle. The only response noted was development of a very small callus and of wound periderm. Another lot was similarly punctured but inoculated since the trident needle was dipped into a culture of *Agrobacterium tumefaciens* grown on agar (University of Chicago strain S 5–6). These plants responded by formation of the neoplasm which is known as crown gall and which, as pointed out by SMITH *et al.* in 1911 (**9**), possesses the essential characteristics of cancerous animal tissues.

Comparative manometric determinations of the oxygen uptake of the normal and neoplastic tissues of these plants, begun September 20, 1943, and continued until late December, showed, as had experiments done in the summer (**6**), that on a fresh-weight basis the oxygen uptake of the tumorous tissue was higher than that of the normal tissue.

The results reported in table 1 were obtained in tests done 50–60 days after puncture and inoculation (the plants were 25 days old when inoculated). By this time the plants had begun to flower, and the tumors had ceased to grow. Some tumors were cracking and showed both superficial and internal necrosis. Care was taken not to use any necrotic tissue in the tests. During the last 20 days the plants received supplementary illumination from incandescent bulbs.

TABLE 1

OXYGEN UPTAKE OF TOMATO TISSUES AS INDICATED

Tissue	Gas phase	"Saturating" light	O_2 uptake rate as % of control	% O_2 uptake mediated by		
				Fe	Cu	"Residual"
Control hypocotyl, control internode, and bisected internode	Air	±	100.0	32.8	34.0	33.2
	CO*	−	47.0			
	CO	+	72.8			
Crown-gall tissue of hypocotyl	Air	±	100.0	12.7	77.3	10.0
	CO	−	29.4			
	CO	+	38.3			
Auxin-tumor tissue of internode	Air	±	100.0	40.8	36.7	22.5
	CO	−	42.0			
	CO	+	70.6			

* 95% CO: 5% O_2 at 1 atm.

Indoleacetic acid (IAA), a growth substance present in normal tissues and, in excessive amounts, in crown-gall tissues (**5**), presumably plays a role in normal growth and development when present in "physiological" concentrations but inhibits normal, or stimulates abnormal, growth and even kills cells when present in abnormal concentrations (**4**). In mid-November, 1943, some control plants of the same planting were used to determine the rate of oxygen uptake of normal

internodes, of bisected internodes, and of the tumor developed on the cut surface of bisected internodes after application of a lanolin paste containing 3% IAA. Massive tumors developed on the wound surface and directly below the site of application. The auxin-induced neoplasms were used in tests when 2–5 weeks old.

In 1950 these experiments, excepting those with auxin tumors, were repeated with younger tissues. The normal control plants were used 30 days after germination. The plants were inoculated when they were 17 days old, and the galls were sectioned and tests run 13 days later (fig. 1).

All manometric tests were made with standard Warburg vessels at 25° C. A buffer solution, as follows, was used: potassium phosphates, 0.01 *M;* $CaCl_2$, 0.005 *M;* $MgSO_4$, 0.002 *M;* pH, 6.0. Three milliliters of this buffer were placed in the main compartment of the vessels, and 0.3 ml. of 15% potassium hydroxide was added to the center cup. The middle portions of the punctured hypocotyls and tumors were sectioned transversely into thin slices which were weighed and placed without washing into the buffer in the vessels. In another series of tests transverse sections were cut from the intact internode, from the stump of the bisected internode, and from the IAA-induced tumors of the bisected internodes. The lanolin paste was removed before sectioning. To inhibit the gas exchanges involved in photosynthesis, 0.15 ml. of *M*/50 hydroxylamine HCl was added to the buffer in the vessels according to the method used by GAFFRON (1). His paper lists the pertinent earlier literature on this method. Ample preliminary tests demonstrated that a concentration of 0.001 *M* hydroxylamine completely inhibits photosynthesis in slices of the control internode and of the crown-gall and auxin neoplasms without disturbing the oxygen uptake of these tissues.

To determine the relative roles played by iron- and copper-containing enzymes in the oxygen uptake of the tissues under test, the carbon monoxide–inhibition methods introduced by WARBURG (10) with intermittent illumination were used. It was assumed that there would be no difference in respiratory intensity of unwashed tomato slices when the pO_2 was decreased from 0.21 (air) to 0.05 (5% O_2). Fortunately, this assumption proved valid when tested experimentally. According to the recent work of MITCHELL *et al.* (7), this was not the case when the tomato-stem tissue slices were washed before testing. In the present tests sufficient carbon monoxide was added to each vessel to give a gas phase containing 5% O_2 and 95% CO.

A special water bath with a glass rear wall and glass bottom was used in a darkroom so that the Warburg vessels could be illuminated at will with a beam of light from a 200-watt projection lamp. This lamp provided light of "saturating" intensity. The second series of tests in 1950 used filtered light of saturating intensity with a maximum transmittance at 430 mμ. In all tests manometric readings were made in the dark at 15-minute intervals for 1 hour in air with and without hydroxylamine in the buffer. Then the mixture of CO and O_2 was run into the proper vessels. Readings were resumed in the dark, and after the second 15-minute reading the vessels were illuminated and two 15-minute readings made. This procedure of alternating light and dark readings was repeated for 1 hour.

WARBURG's data (10) to the effect that 95% carbon monoxide inhibits approximately 70% of the iron-mediated

oxygen uptake in the dark with reversal of inhibition in the light, and the data of KUBOWITZ (2) and LEVY and SCHADE (3) that 80% of the copper enzyme-mediated oxygen uptake in the dark is inhibited with no reversal of inhibition in light, were used to calculate the percentages of oxygen uptake mediated respectively by iron- and copper-containing enzymes.

Even if the calculated percentages of mediation of the iron, copper, and residual systems based on values obtained from other plant systems (2, 3, 10) are

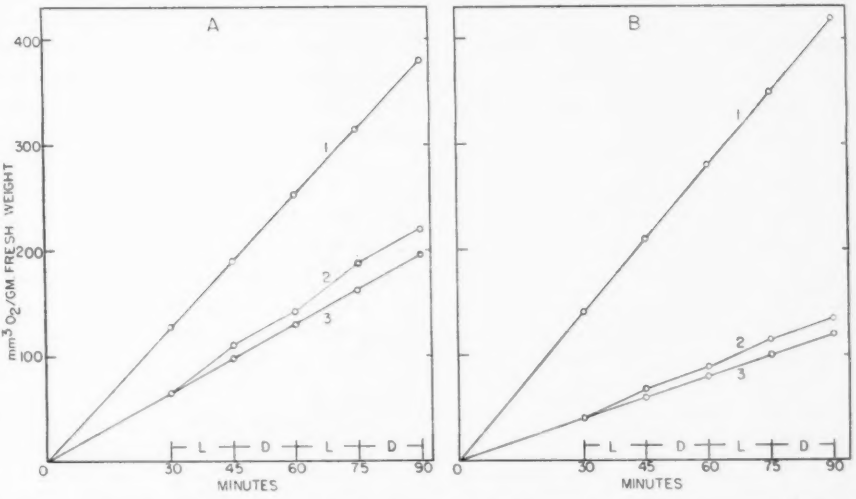

FIG. 1.—Oxygen uptake of control (*A*) and crown gall (*B*) of tomato hypocotyl tissues. *1*, 95% N_2:5% O_2. *2*, 95% CO:5% O_2 with alternating light (*L*) of saturating intensity and dark (*D*) periods. *3*, 95% CO:5% O_2 in dark.

Results

The data and calculated values are given in table 1 and figure 1. They both show that the carbon monoxide inhibition of oxygen uptake was only partially reversed by saturating light. From these results it is concluded that both iron and copper enzymes are operative in the oxygen uptake of all the tomato tissues tested. In all cases there also was an oxygen uptake (designated "residual") which is not mediated by either iron or copper enzymes. The calculated data show that the percentages of oxygen uptake mediated by iron, copper, and "residual" enzyme systems are the same for tissues of the normal hypocotyl, normal internodes, and the stumps of bisected internodes at the various stages of development tested.

not quantitatively applicable to tomato tissues, it is evident that crown-gall and IAA-induced tumors differ from normal tissues and from each other in the extent of the role played in them by these enzyme systems. In the crown-gall tissue, the shift is in favor of the copper enzymes at the expense of both iron and residual systems, confirming the gross findings of NAGY *et al.* (8) to the effect that tyrosinase activity is enhanced in tomato crown-gall tissue. In the auxin tumors, on the other hand, the percentages of oxygen uptake mediated by iron

and copper enzymes were increased at the expense of the residual system.

Discussion

These experiments were not designed to determine whether there is a detectable effect on respiration immediately after wounding and bisection of the tomato stem or during the healing process. They were done when wound-healing had been completed, in the hope that a steady metabolic state would have been attained. The findings show that tissues of the stump of the internode used oxygen at the same rate and via the same pathways as those of the intact internodes.

However, the injuries done by (*a*) wound application of a presumably hypernormal concentration of auxin (IAA) and (*b*) introduction of *Agrobacterium tumefaciens* into the tissues are sufficient not only to result in an increase of the rate of oxygen uptake of the neoplastic tissues over the normal tissues (on a fresh-weight basis) but also to alter quantitatively the relative roles of iron, copper, and residual enzyme systems in mediating oxygen uptake in these tissues relative to normal tissues. Whether these changes are related as cause and/or effect to neoplastic growth remains to be determined. It also remains to be established whether these findings apply to auxin and crown-gall neoplasms at all stages of their development. If this should be the case, these findings may point a way to a better understanding (*a*) of the relation of respiration in plants to normal and abnormal growth and development and (*b*) of the mechanism whereby auxins affect the metabolism of plant cells.

Summary

1. Carbon monoxide–inhibition studies were made of the oxygen uptake of normal tissues and crown-gall tissues of the hypocotyl and internodes and of auxin-induced tumors of the internodes of tomato (*Lycopersicon esculentum*).

2. The results indicate that iron and copper enzymes and enzymes not yet identified mediate oxygen uptake in the tissues studied.

3. The percentages of oxygen uptake mediated by these enzymes are the same for tissues of the normal hypocotyl, internodes, and stumps of bisected internodes at two stages of development.

4. In the crown-gall tissues a percentage shift occurred in favor of the copper enzymes at the expense of both the iron and the residual enzyme systems. In the auxin tumor the percentage of oxygen uptake mediated by both iron and copper enzymes was increased at the expense of the residual systems.

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BIOSYNTHESIS OF RADIOACTIVE NICOTINE[1]

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Introduction

The recent application of radioactive tracer techniques to biological problems has greatly increased our knowledge of many aspects of the biological sciences.

One of the important uses of isotopes is their introduction into the molecules of chemical compounds which are of great value in clinical medicine. Many of these substances have a complex chemical structure and at present cannot be synthesized chemically; they must be extracted from plants. In order to introduce a radioactive isotope into such molecules, the method of biological synthesis must be employed. By means of this technique the radioactive isotope (in our work C^{14}) is introduced into the plant as a simple precursor, $C^{14}O_2$, from which the plant synthesizes the desired compound. After allowing time for the formation of sufficient amounts of the desired compound, the plant material is processed for the purpose of isolating and purifying the resultant radioactive drug.

The experiments presented here deal with the biosynthetic preparation of a radioactive form of the main alkaloid in tobacco plants—nicotine. This work is part of an extensive research project, the main objective of which is the preparation by biosynthesis of radioactive drugs which are of value in medicine (**5**).

Experimentation

PLANT GROWTH.—Tobacco plants (*Nicotiana rustica*) were grown from seed in the greenhouse and transplanted to soil in large flats when they had developed an adequate root system. After an additional period of about 1 month, they were transferred, in soil, to 6-inch glazed pots. Each plant was then placed in a cylindrical, Pyrex battery jar, 2 feet tall by 12 inches in diameter, and the top covered with a circular piece of plate glass, the whole made airtight with calking compound. A water manometer was attached to the system to test adequacy of seal. Inlet facilities for the introduction of carbon dioxide and nutrient solution into this closed system were provided. The temperature was maintained between 65° and 75° F., and illumination was provided by a system of white fluo-

[1] This work was done under a contract between the Atomic Energy Commission and the University of Chicago. It was aided in part by grants from the Life Insurance Medical Research Fund and the Dr. Wallace C. and Clara A. Abbott Memorial Fund.

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[3] These studies are taken from a dissertation submitted to the University of Chicago in partial fulfilment of the requirements for the degree of Doctor of Philosophy, September, 1950.

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rescent lights, giving an intensity at the plant of 300 foot-candles. The light period per day varied from 8 to 12 hours.

RADIOACTIVE CARBON DIOXIDE ($C^{14}O_2$).—Radioactive barium carbonate was obtained from the Atomic Energy Commission National Laboratory at Oak Ridge, Tennessee. Depending on the total activity desired, an amount of radioactive carbon was weighed and placed in the barrel of a 100-ml. syringe. A known amount of normal barium carbonate was also added to the radioactive carbonate. The syringe was fitted with a three-way valve, and another syringe of 10-ml. capacity was fitted to the valve. The plunger of the larger syringe was greased to insure against leakage. A lactic acid solution was placed in the small syringe, and both plungers were replaced. By use of this arrangement it was possible to add the contents of the small syringe to the large syringe by opening the proper valve without any loss of the generated radioactive carbon dioxide. A needle was attached to the large syringe, and the radioactive carbon dioxide injected into the sealed plant system. The tobacco plant was allowed to grow in this radioactive environment for a period of 4–6 weeks, during which time the photosynthetic mechanisms incorporated considerable amounts of the radioactive carbon dioxide into its carbon-containing constituents. Samples of gas from the inclosed radioactive atmosphere were removed from time to time and were analyzed for radioactivity. In this way it was possible to check on the continued viability of the plant, since a day-to-day decrease in the total radioactivity of the inclosed atmosphere could only result if the plant was actively photosynthesizing. At the conclusion of the period of time necessary for the uptake of the desired amount of radioactive carbon, the plants were harvested and dried to constant weight at room temperature before being subjected to extraction procedures.

EXTRACTION AND PURIFICATION.—The nicotine was extracted from the plants by a modification of the usual steam-distillation procedure. The dried plant material was homogenized and steam distilled from a dilute alkaline (0.1 *N* NaOH) medium. The official procedure (1) employs a stronger alkaline medium containing NaCl. A dilute (0.1 *N* HCl) acid solution was used to collect the distillate. In this way, 150–200 ml. of distillate were collected, since preliminary experiments on the rate of nicotine distillation under these conditions showed that the distillation was practically complete at this stage. From time to time the distillation apparatus was tested by distilling known amounts of nicotine. One hundred per cent recovery was consistently attained. The distillate from tobacco plants was clear and colorless and suitable for spectrophotometric analysis. The ultraviolet absorption curves of these distillates were found to be identical with those obtained using standard nicotine solutions. By means of radioactive methods of analysis, however, it was found that the decrease in radioactivity upon further purification of these distillates was greater than the decrease in nicotine content as determined spectrophotometrically. This finding indicated the presence in the distillate of spectrophotometrically inert, radioactive impurities. Further purification was therefore carried out on all radioactive distillates, since an essential condition of metabolic studies with radioactive materials is that any radioactivity found in the body of an organism must be derived only from the radioactive substance whose metabolism is being studied. Table 1 presents the losses with purification of three radio-

active nicotine solutions, as measured by spectrophotometric and radioactive techniques.

The purification procedure used consisted of a silicotungstic acid precipitation of the nicotine in the distillate followed by successive extraction of the precipitate with alkali, ether, and acid. was analyzed spectrophotometrically, and the absorption curve was identical with those obtained using known nicotine solutions.

SPECTROPHOTOMETRIC ANALYSIS.—In an effort to find an analytical method for nicotine that would eliminate the disadvantage of sample destruction that is

TABLE 1

PURIFICATION DATA OF THREE RADIOACTIVE NICOTINE SOLUTIONS AS OBTAINED BY SPECTROPHOTOMETRIC AND RADIOACTIVE ANALYSES

Before purification				After purification			
Vol. (ml.)	Total amount of nicotine in solution (spect.) (mg.)	Total radioactivity of solution (c.p.m. $\times 10^6$)	Calculated activity of nicotine (c.p.m./mg.)	Vol. (ml.)	Total amount of nicotine in solution (spect.) (mg.)	Total radioactivity of solution (c.p.m. $\times 10^6$)	Calculated activity of nicotine (c.p.m./mg.)
163	9.37	3.67	392,000	11.0	8.92	2.76	310,000
194	10.40	3.91	376,000	10.8	9.32	3.09	332,000
185	8.31	4.63	557,000	9.7	7.68	3.85	501,000

PURIFICATION RECOVERY VALUES

Spectrophotometric Method (%)	Radioactive Method (%)
95.2	75.2
89.6	79.1
92.4	83.1
Average 92.4	79.1

The concentration of silicotungstic acid used and the pH conditions of the precipitation were those employed by SPIES in his method for the microdetermination of nicotine (**8**). The choice of solvents for the extraction of nicotine from the nicotine silicotungstate precipitate was dictated by the known solubility characteristics of nicotine as an alkaloid. Several small portions of each of the solvents were used to minimize incompleteness of extraction. The final purified acid extract

inherent in the usual colorimetric procedures, the Beckman ultraviolet spectrophotometer was used. Nicotine was found to possess a characteristic absorption curve in the ultraviolet region between 220 and 280 mμ and a minimum at 230–232 mμ; this absorption is appreciable in concentrations as low as 5 μgm/ml (fig. 1). Using the peak of absorption at 260 mμ for the quantitative determinations and for analyzing increasing concentrations of nicotine in so-

lution, a straight-line relationship between concentration and optical density was found to hold, indicating the applicability of Beer's law to the method (fig. 2). The identity of the absorption curves obtained with the tobacco-plant

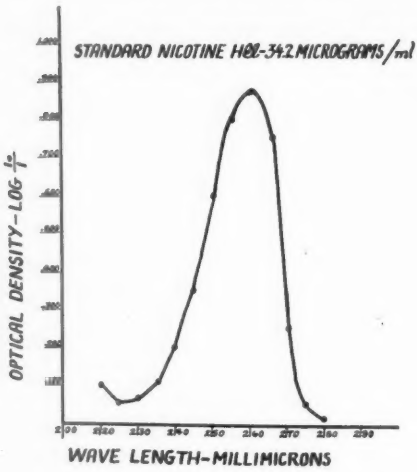

FIG. 1.—Ultraviolet absorption curve of nicotine between 220 and 280 mμ.

distillates with the absorption curves of known nicotine solutions indicated the applicability of the method to the determination of nicotine concentrations in radioactive tobacco extracts.

RADIOACTIVE ANALYSIS.—Quantitative determinations of radioactivity were made using a highly sensitive, internal-flow, helium gas counter (7). An aliquot of the radioactive distillate or purified solution was evenly spread over a 10-sq.-cm. area of a weighed copper disk. This liquid was carefully evaporated to dryness on a turntable, using an infrared lamp and a warm-air blower. The low dry weight made self-absorption corrections unnecessary. By means of this technique highly reproducible results of duplicate determinations were obtained, and various dilutions of a given radioactive nicotine solution gave results that closely corresponded to the dilution factor employed. Counting time was of sufficient length to give a standard error of 3% or less. It was found necessary to acidify all radioactive solutions before evaporation, because of the volatility of nicotine from neutral or alkaline solutions on heating. The loss of nicotine when evaporated from neutral solutions under the conditions described above averaged 10%, while the loss from weakly alkaline solutions averaged 25%.

TESTS FOR IMPURITIES.—The possible presence in plants of impurities that might absorb in the ultraviolet region used for the analysis of nicotine was tested by using a plant species closely related to tobacco. The plant used was

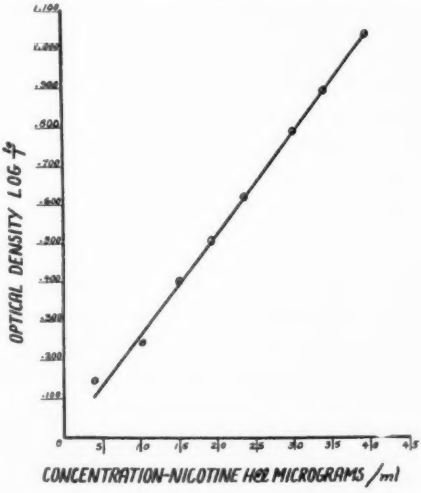

FIG. 2.—Relation between concentration and optical density at 260 mμ of known nicotine solutions.

Atropa belladonna, which is a member of the same family (Solanaceae) as tobacco but does not contain nicotine. This plant was harvested, dried, and steam distilled under the identical conditions used for the extraction of nicotine. The clear,

colorless distillate obtained was analyzed spectrophotometrically in the ultraviolet region which was used for nicotine analysis and was found to exhibit insignificant absorption of a nonspecific type.

It has been recently emphasized that nornicotine, the pyrollidine N-demethylated derivative of nicotine, is present in significant amounts in many species of tobacco (3). The official method for the determination of nicotine in tobacco (1) is not designed to separate these closely related alkaloids, and in consequence the inclusion of the nornicotine in the total nicotine value could represent a considerable error in the determination. Since the purpose of the present study was the preparation of radioactive nicotine in pure form, the elimination of nornicotine was essential. BOWEN and BARTHEL found (3) that the nornicotine content as well as the nicotine-nornicotine ratio varied widely in different species of tobacco plants. *Nicotiana rustica* (Brasilia), the species primarily used in our experiments, was found to possess a very low nornicotine content and a very high nicotine-nornicotine ratio. Furthermore, these workers found that under certain conditions the property of distillability with steam is markedly different between the two alkaloids. Nornicotine was found to distil very slowly from weakly alkaline solutions in comparison with nicotine. Our distillation experiments were designed to take advantage of this difference. The combined precautions of selecting the appropriate species of tobacco plant and the optimum distillation conditions were sufficient to remove nornicotine as an impurity in the preparation of radioactive nicotine, as proved by spectrophotometric and by melting-point data.

Although nornicotine displays absorption capacity of the same degree as does nicotine in the ultraviolet and also has its peak of absorption at 260 mμ, the shape of the absorption curve, examined as a whole, differs markedly from the nicotine curve. Since the absorption properties of two or more compounds in the same solution are additive, provided, of course, that no chemical reaction has taken place, the presence of nornicotine, in any but trace amounts, as an impurity would alter the shape of the typical nicotine curve and could, therefore, be detected. The identity of the absorption curves obtained using tobacco distillates with the absorption curves obtained with solutions of pure nicotine constitutes good evidence of the absence of nornicotine or any other substances absorbing in this ultraviolet region. The spectrophotometric evidence for the absence of nornicotine as an impurity is further supported by the results of the melting-point determinations. Nornicotine forms a picrate whose melting point is 191° C. (corr.), while the picrate of nicotine melts at 217°–218° C. (corr.). The picrate of the purified plant material had a melting point of 218° C. (corr.). The picrate of a pure nicotine solution was prepared, and a mixed melting-point determination carried out with the unknown picrate. No depression of the nicotine melting point was observed.

IDENTIFICATION OF NICOTINE.—The following considerations are offered as evidence that the radioactive material obtained from tobacco plants grown in an atmosphere of radioactive carbon dioxide is nicotine:

a) The identity of its spectrophotometric absorption curve with that obtained by using known nicotine solutions.

b) Its precipitation with silicotungstic acid and its solubility properties in alkaline, ether, and acid solutions.

c) Its property of being distillable with steam from weakly alkaline solutions but not from acid solutions.

d) The melting point of the picrate of the purified material is identical with that of nicotine picrate. The mixed melting point of nicotine picrate and the picrate of the purified material showed no depression from the melting point of known nicotine picrate.

e) The purified material, when injected into mice in doses corresponding to those used with known nicotine, gave the typical nicotine effects of initial excitement and convulsions followed by secondary depression and death.

f) When injected into the ventral lymph sac of frogs, the purified material, in doses equivalent in amount to known nicotine, elicited the peculiar hind-limb flexor reflex highly characteristic of nicotine (6).

TABLE 2

RATE OF FORMATION OF NICOTINE IN LEAVES OF GROWING TOBACCO PLANTS

Weeks after transplantation	Nicotine in leaf, percentage dry weight	
	Sample I	Sample II
	N. rustica (Brasilia)	
4	0.45	.....
7	0.70	0.66
10	0.90	0.92
12	0.94	0.96
15	1.10	1.06
18	1.22	1.23
	Johns Bros.	
8	1.19	1.23
12	1.38	1.40
16	1.62	1.67
20	1.87	1.97

RATE OF FORMATION OF NICOTINE IN GROWING PLANTS.—The rate at which growing tobacco plants form nicotine was studied by determining the nicotine content of leaves taken from two strains of tobacco plants at various stages of growth (table 2). A slow but definite accumulation of nicotine in the leaves was found. This is in agreement with the generally held views of nicotine formation by tobacco plants—that nicotine is synthesized in the roots and is transported via the stems to the leaves, where it continues to accumulate as the plant grows (4).

TABLE 3

RELATION BETWEEN AMOUNT OF RADIOACTIVE CO_2 GIVEN YOUNG TOBACCO PLANTS AND RADIOACTIVITY OF EXTRACTED NICOTINE

Microcuries of $C^{14}O_2$ injected	C.p.m./mg. of nicotine extracted from plant	C.p.m./mg nic./μc. $C^{14}O_2$
2	1,200	600
50	23,500	470
100	53,000	530
250	125,000	500
600	310,000	518
900	501,000	556
Average	.....	529

The highest rate of nicotine formation apparently occurs during the early growth period. The highest radioactivity of extracted nicotine would, therefore, be expected when young plants are exposed to radioactive carbon dioxide atmospheres. This was found to be the case. When young and old plants were exposed to equal amounts of $C^{14}O_2$ for the same period of time, the activity of the nicotine extracted from the young plants was frequently as high as 200% greater than that of the older plants.

RADIOACTIVE NICOTINE.—A summary of the radioactivity of the nicotine obtained from young tobacco plants ex-

FIGS. 3–4.—Radiation effects on young, growing tobacco plants. Fig. 3 (*above*), plant on left received 900 microcuries of $C^{14}O_2$ over 2-week period at early growth stage. Plant on right was grown under identical conditions but received normal CO_2 instead of radioactive CO_2. Fig. 4 (*below*), three leaves on right were from plant in figure 3 that received radioactive CO_2; three leaves on left were from control.

posed to various amounts of $C^{14}O_2$ is presented in table 3. When the values are calculated in terms of the activity of the extracted nicotine per microcurie of $C^{14}O_2$ injected, the values obtained are roughly equivalent and average 529 c.p.m./mg. of nicotine/microcurie of C^{14} injected as $C^{14}O_2$. This rough constant is indicative of the efficiency with which the administered radiocarbon is incorporated into the nicotine molecule by the biosynthetic process of the tobacco plant. No differences in the total nicotine content of normal and radioactive plant materials were found, indicating that the levels of radioactivity achieved in these experiments did not interfere with the ability of the tobacco plant to synthesize nicotine.

RADIATION TOXICITY TO PLANTS.—In an attempt to determine what, if any, gross effects could be demonstrated in tobacco plants receiving large amounts of radioactive carbon dioxide, three young tobacco plants of identical backgrounds were selected. These plants were sealed in battery jars according to the procedure described. One of the plants was used as a control, and the other two were given radioactive carbon dioxide in divided doses over a period of 2 weeks until 900 microcuries had been administered to each plant. All three plants were allowed to grow for an additional 3 weeks, at which time they were removed from the sealed system and examined. Marked morphological differences between the control and radioactive plants were observed. The radioactive plants were less robust in appearance (fig. 3), and, although the number of leaves on these plants was greater than on the control plant, the size of the individual leaves was reduced. Also, the leaves of the radioactive plants were much narrower (fig. 4), and were a darker shade of green than those of the control.

Leaf samples from tobacco plants receiving radiocarbon were collected and placed in a formalin fixative solution. Histological studies of these samples were carried out by Professor J. M. BEAL of the Department of Botany of the University of Chicago. His observations are reported in a preliminary paper (2). The main histopathological effects which he attributed to C^{14} radiation toxicity are shrinkage of the protoplasts, degeneration and reduction in size of the plastids, and reduction in size and number of the intercellular spaces. The presence of dead and dying cells and alterations in the pattern of tissue differentiation in the leaf, particularly in the upper epidermis and the palisade parenchyma, were also noted.

Summary

1. The biosynthetic approach to the preparation of labeled compounds for metabolic studies can be used where chemical synthesis is not possible or practical.

2. Using the photosynthetic mechanism of the tobacco plant to incorporate radioactive carbon dioxide into its constituents, radioactive nicotine has been prepared. The highest activity of radioactive nicotine obtained was 501,000 c.p.m./mg. (0.3503 μc./mg.).

3. A highly convenient and sensitive spectrophotometric method has been developed for the analysis of nicotine in tobacco extracts.

4. The identity of the extracted and purified material as nicotine is evidenced by spectrophotometric and by melting-point data, by precipitation and solubility characteristics, and by biological action on mice and frogs.

5. The value of the specific radioactivity of extracted nicotine per unit of radio-

activity administered to the plant is roughly constant when different amounts of radioactivity are given to young tobacco plants. This value averaged 529 c.p.m./mg. of nicotine/microcurie of radiocarbon injected as $C^{14}O_2$.

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THE GENETIC BASIS OF WOOLLY DEGENERATION IN NEUROSPORA CRASSA[1]

T. C. SHENG

Introduction

The gradual change of certain morphological or physiological characters of a bacterial or fungal culture during successive transfers is usually known as physiological degeneration. In general, pathogenic bacteria tend to lose virulence gradually when kept in cultures on artificial media. It has been reported that the penicillin production in certain strains of *Penicillium notatum* always decreases with successive transfers (**14**). In extreme cases cultures are known that can survive only a limited number of transfers after their isolation from natural habitats as in certain strains of Actinomycetes studied by KRASILINIKOV (**13**). In *Neurospora* a similar kind of decline called natural death (*nd*) has been observed in a mutant strain which, nevertheless, may be maintained in a balanced forced heterocaryon for apparently unlimited transfers and be re-isolated in a rejuvenated condition whenever desired (**10**).

One of the common types of physiological degeneration in fungi is known as woolly degeneration (**12**). It is characterized by a gradual reduction in the conidiation of a culture of fungus on successive transfers so that the culture may finally appear woolly. Dual phenomenon (heterocaryosis) of true-breeding mycelial elements in conidial cultures is of wide occurrence among imperfect fungi (**4**) including *P. notatum* (**5**) and also in *Neurospora* (**11**). Furthermore, heterocaryons of morphological and biochemical mutants are common (**1, 7**), and spontaneous mutation and selection of a biochemical character have been known to occur in cultures of *Neurospora* (**8**). It

[1] From the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena. This work was done during the tenure of a Public Health Fellowship sponsored by the National Cancer Institute, Federal Security Agency.

is therefore the prevailing notion that "the change in the organism that leads to physiological degeneration is, in the final analysis, of genetic origin, the change being an inherent feature of the subculture" (2). Reduction in conidiation also accompanies the ever decreasing growth potential of the *nd* mutant just mentioned. Nevertheless, the degeneration has been shown to be not due to mutation and selection (10). In this paper a genetically determined woolly degeneration in *Neurospora* which is likely to be of the same nature will be described.

TABLE 1

CONIDIAL COUNTS OF A WD AND A NON-WD STRAIN ON SUCCESSIVE TRANSFERS IN 25° C. DARKROOM WITH INTERMITTENT ILLUMINATION OR UNDER CONSTANT ILLUMINATION

CULTURAL CONDITION	NUMBER OF TRANSFER	CONIDIA PER TUBE	
		wd	non-*wd*
In darkroom	1	458×10^5	4000×10^5
	2	64×10^5	400×10^5
	3	26×10^5	300×10^5
	4	12×10^5	200×10^5
	5	2.6×10^5	468×10^5
	6	6×10^5	1200×10^5
	7	0.2×10^5	920×10^5
	8	0	700×10^5
	9	0	820×10^5
	10	0	
	11	0	
	12	0	
Under illumination	13	2080×10^5	
	14	1520×10^5	
In darkroom	15	3.4×10^5	
	16	1×10^5	
	17	0	

Experimentation and discussion

It was noticed that there was no difficulty in obtaining conidia of *Neurospora crassa* for inoculation when certain cysteine-requiring cultures were freshly isolated from a cross of a cysteineless mutant (84605A) to an albino wild-type strain (15300a). On successive transfers they gave rise to less and less conidia. It was observed in no less than ten asci that this character was determined by a single Mendelian gene, which is accordingly referred to as woolly degeneration (*wd*). There is no striking difference macroscopically in the freshly isolated (first transfer) cultures of *wd* and non-*wd* strains, but classification of the two types was in most cases unambiguous in the second transfer, especially when the cultures were kept in darkness (see table 1). Among a hundred randomly isolated spores there was none that showed a recombination of the cysteine-requiring and the woolly-degeneration character. This gene is therefore closely linked to the cysteineless gene which has been localized on the second chromosome

near the gene riboflavin-less 51602 (**6, 9**).

As conidiation in *Neurospora* is very sensitive to cultural conditions, especially illumination, very marked woolly degeneration occurs only at relatively low temperature (25° C.) and in darkness or under weak illumination. An extreme case of woolly degeneration is shown in the first column, and a non-*wd* culture from a cross involving two wild types is shown in the second column of table 1. All cultures were maintained on FRIES medium agar with 0.2 mg. L(-)tyrosine and 0.5 mg. L(-)cysteine per 20 ml. of medium (**9**) in 16 × 160-mm. tubes each filled with 8 ml. of agar medium and so slanted that the agar surface was 9–10 cm. long. The 8-day-old cultures were washed thoroughly with a convenient amount of water ranging from 4 to 80 ml., and the counts were made with a hemocytometer. The average difference between ten duplicate counts was 17%. When no conidium was visible on the hemocytometer, careful search was made on a sample of the washing together with mycelium under the microscope. No free conidium could be found in those transfers marked zero, though beaded hyphal tips were occasionally observed. On putting the thirteenth transfer under constant illumination with a 14-watt "daylight" lamp 50–60 cm. from the culture, the conidial count rose up suddenly beyond that of the original culture kept in the darkroom. The conidial count remained on a high level as long as the transfers were kept under illumination. It decreased stepwise as before but even more abruptly when the culture was returned to the darkroom (table 1).

There could be two alternative explanations for this observation. First, mutation from the conidial to the mycelial character occurs in the single-spore cultures originally conidial in nature, and the latter may gradually be selected and may finally overgrow the former. Second, genetically the cultures remain unchanged, but, owing to some unknown physiological cause, the capacity for conidiation gradually becomes exhausted. The second hypothesis is favored for the following reasons and for additional findings.

First, according to the mutation-and-selection hypothesis, a culture after being mycelial for several transfers should contain mainly, if not entirely, mycelial nuclei. However, in a cross of the twelfth transfer of the *wd* strain, which was mycelial, to a non-*wd* strain, all the *wd* cultures freshly isolated and tested under the same conditions had high conidial counts comparable with the original (mean 718×10^5, range $540–1200 \times 10^5$) among eleven single-spore cultures from six whole asci. The hypothesis of mutation and selection, therefore, seems to be improbable, unless we make the following unlikely assumptions: (*a*) that fertilization is highly selective and (*b*) that the mycelial nuclei are so dominant that even in the highly degenerated culture the conidial nuclei still far outnumber the mycelial ones, though the culture itself appears mycelial.

Second, even in the non-*wd* cultures there was less conidiation especially in the second transfer (table 1). This has been observed altogether in twenty single-spore cultures, *wd* and non-*wd*. It is unlikely that mutation occurs in every case. It is rather reasonable to think that this is a general physiological property, which is more exaggerated in the presence of the *wd* gene.

The degeneration described is therefore very likely not mutational in nature, though determined by a single gene. The

degeneration may not be comparable with those known in other fungi, and similar degeneration may be due to mutation and selection in other cases or in other organisms as claimed (3, 5). A distinction between two types of degeneration is important: degeneration that is due to mutation and selection and that due to physiological causes, be it determined by a single gene or not. The former may be called apparent physiological or genetic degeneration and the latter genuine physiological or simply physiological degeneration.

Summary

A gene that is responsible for woolly degeneration in *Neurospora* is described. The degeneration has been shown to be due to some kind of physiological cause rather than mutation and selection.

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GLUTAMIC ACID IN SUGAR BEETS INCREASED BY ZINC DIMETHYLDITHIOCARBAMATE[1]

JESS L. FULTS, MERLE G. PAYNE, JOHN O. GASKILL, LUCILE R. HAC, AND ALBERT C. WALKER[2]

Introduction

Numerous investigations have shown that ethyl phenylcarbamate (urethane) has c-mitotic effects on plant and animal cells (**1, 4, 5, 6**). It has also been shown that ethyl carbamate, zinc dimethyldithiocarbamate (du Pont and Co.—Zerlate), and sodium ethylene–bisdithiocarbamate (Rohm and Haas Co.—Dithane D-14) are superior to 2,4-dichlorophenoxyacetic acid (2,4-D) in the standard etiolated-pea test for primary growth regulation (**2**). The selective effect of ethyl phenylcarbamate and related aryl carbamates and thiocarbamates against certain cereals and other grasses has suggested their use as selective herbicides for weedy grasses (**9**).

Preliminary field tests with plant spray applications of zinc dimethyldithiocarbamate and other growth-regulating chemicals were made on sugar beets near Ault, Colorado, during 1946. These tests were made to determine effects on beet yields, sucrose percentages, sucrose per acre, and glutamic acid percentage. Results showed no significant differences in either root yield, sucrose percentage, or sucrose per acre. The glutamic acid determinations indicated a 100% increase resulting from Zerlate treatment. Actual average increase was from 0.028% in the controls to 0.056% in the treated samples. The data on sucrose were considered critical, but not the glutamic acid data, as they were based on too few samples. However, the results were encouraging, and a detailed critical test was made in 1948. Since treatments with 2,3-dimethylphenoxychloroacetic acid in 1946 also indicated a resultant increase in glutamic acid, this compound was included in the 1948 test. The results are here reported.

Material and methods

A commercial variety of sugar beets, G.W. 59, was grown under irrigation in the usual way with 20-inch spacing between rows and approximately 12-inch spacing in the row. The crop was planted May 4–5 and harvested November 2–3, 1948. Soil fertility was good and moisture adequate, but defoliation by hail on August 2 caused reduction in potential tonnage and probably also in potential sucrose percentage. The foliage recovered satisfactorily, regaining normal size by

[1] This is a contribution of the Botany and Plant Pathology Section and Chemistry Section, Colorado Agricultural Experiment Station; of the Division of Sugar Plant Investigations, Bureau of Plant Industry, Soils and Agricultural Engineering, U.S. Department of Agriculture, Fort Collins, Colorado; and of the Research Division of the International Minerals and Chemical Corporation, Woodland, California. Published with the approval of the Director, Colorado Agricultural Experiment Station as Scientific Series Paper no. 350.

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about September 1. The seven treatments were as shown in table 1. Plants were treated on July 20 and September 8, 1948. At each date the test chemicals were suspended separately in a quart of water and sprayed by means of a hand sprayer equipped with a no. 2 flat fan-shaped nozzle and operated at a pressure of 20 pounds per square inch. The quart of spray was applied uniformly over the entire plot (four rows, 27 feet long) in the case of zinc dimethyldithiocarbamate. In the case of 2,3-dimethylphenoxychloroacetic acid the two inner rows of each plot were sprayed. The acre rates for the two chemicals were approximately 60 gallons and 120 gallons, respectively. The rates of application for Zerlate (70% zinc dimethyldithiocarbamate, E. I. du Pont de Nemours & Co.) were 7.86 pounds, 3.93 pounds, and 1.97 pounds per acre on each of the two treatment dates. The usual rates of application on sugar beets when used as a fungicide are 1.5–2 pounds per 100 gallons of water per acre per application. The rates of application for the 2,3-dimethylphenoxychloroacetic acid were 0.4 pound, 0.2 pound, and 0.1 pound per acre. (This latter compound was prepared by D. M. GRIFFIN of the Chemistry Department of Colorado A. and M. College.)

TABLE 1

EFFECT OF TWO SYNTHETIC GROWTH-REGULATORS ON SUGAR-BEET YIELD, SUCROSE PERCENTAGE, GROSS SUCROSE, AND GLUTAMIC ACID PERCENTAGE
(Ault, Colorado, 1948)

TREATMENT NUMBER	CHEMICAL		TONS ROOTS PER ACRE	% SUCROSE	TONS GROSS SUCROSE PER ACRE	% GLUTAMIC ACID		
	Kind	Units*				Actual mean	Mean of logs.†	Geom. mean†
1.....	2,3-dimethylphenoxychloroacetic acid—No. 121	4	13.02	11.56	1.515	0.069	0.8345	0.068
2.....	2,3-dimethylphenoxychloroacetic acid—No. 121	2	15.23	12.48	1.888	.064	.7944	.062
3.....	2,3-dimethylphenoxychloroacetic acid—No. 121	1	14.41	12.00	1.754	.063	.7680	.059
4.....	Zinc dimethyldithiocarbamate—No. 186	4	16.16	12.20	1.984	.082	.8864	.077
5‡....	Zinc dimethyldithiocarbamate—No. 186	2	15.59	12.45	1.937	.084	.9065	.081
6.....	Zinc dimethyldithiocarbamate—No. 186	1	16.52	12.24	2.045	.073	.8231	.067
7.....	None	.....	15.34	12.12	1.871	0.060	0.7933	0.062
L.S.D. (5% point)	.....	.....	3.66	1.06	0.470	.....	0.1177	.....

* One unit of zinc dimethyldithiocarbamate is equivalent to two applications of 1.38 pounds per acre; one unit of 2,3-dimethylphenoxychloroacetic acid is equivalent to two applications of 0.1 pound per acre.

† Statistical analysis performed by latin-square technique, with appropriate adjustment of degrees of freedom, after estimating plot values for a theoretical seventh column and transforming percentage data into logarithms; estimation procedure devised by A. G. CLARK, Professor of Mathematics, Colorado A. and M. College (unpublished).

‡ Owing to loss of a pulp sample, it was necessary to estimate one missing-plot value for each attribute shown except root yield.

The experimental plot design was equivalent to a 7×7 latin square with one column deleted. Plots were four rows wide and 27 feet long. At harvest all roots in the two inner rows of each plot, less $1\frac{1}{2}$ feet at each end, were topped, washed, and weighed for determinations of root yield. Computations were based on total harvested area, i.e., 48 feet of row per plot. A random sample of twen-

ty-five of the harvested roots from each plot was analyzed for sucrose content by a modification of the Sachs-LeDocte method (**8**), and a representative portion of the pulp obtained from each root sample in this process was quick-frozen and shipped in dry ice to Woodland, California, for determinations of glutamic acid.

MEASUREMENT OF GLUTAMIC ACID PERCENTAGE.—Pulp samples were thawed, extracted with isopropanol (70% by volume), and an aliquot of the filtered extract was evaporated almost to dryness under reduced pressure. The noncollodial fraction of the beet thus obtained was hydrolyzed with barium hydroxide and assayed microbiologically for glutamic acid according to the procedures described by HAC *et al.* (**3**).

Results and discussion

None of the differences between control and chemically treated plants was significant in the case of root yield, sucrose percentage, or yield of gross sucrose (table 1), though the data indicate the possibility of detrimental effect of treatment 1, the heaviest application of 2,3-dimethylphenoxychloroacetic acid.

The only important deviations from the control in glutamic acid content were the increases shown for treatments 4 and 5, the 4-unit and 2-unit applications of zinc dimethyldithiocarbamate. The gain for treatment 5 closely approached the 5% point, and that shown for treatment 4 was only slightly lower. If the assumption is made that only the 4-unit and 2-unit dosages of the chemical were effective in bringing about an increase in glutamic acid content, then the average obtained by combining the results from those two treatments may be compared with the average for the control. The difference obtained from such a comparison in the column of logarithms is 0.1032—a figure which, for all practical purposes, may be considered as equivalent to the 5% point (0.1037) applicable to such a mean difference.

These results may have a direct application to the problem of increasing glutamic acid content of sugar beets grown in the northern Colorado area. It is possible that in the results reported here the zinc ion may have been responsible for the effect, but if zinc were a limiting factor, some response would have been expected in the data on beet yield or sucrose. There was no such response in either 1946 or 1948, nor were zinc-deficiency symptoms observed at any time. The effect was probably not a fungicidal or bactericidal one, since the amount of disease in each case was negligible.

The increased accumulation of glutamic acid in beets sprayed with a growth-regulating compound is in agreement with a recent report by RAKITIN *et al.* (**7**). These workers found significant increases in amino nitrogen in potatoes treated with the methyl ester of naphthaleneacetic acid.

Summary

1. The effects of two foliage-spray applications of zinc dimethyldithiocarbamate (Zerlate) and 2,3-dimethylphenoxychloroacetic acid on root yield, sucrose, and glutamic acid content of sugar beets were studied in a replicated experiment near Ault, Colorado, in 1948.

2. An apparent increase in actual mean glutamic acid percentage of harvested roots resulted from treatment by either chemical at all three rates used. Differences in the case of 2,3-dimethylphenoxychloroacetic acid were not considered significant. The difference between the control value and the mean for the medium and high rates of treatment

with zinc dimethyldithiocarbamate was approximately equivalent to the 5% level of significance.

3. No significant effects of treatment were observed for root yield, sucrose percentage, or gross yield of sucrose.

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MOISTURE RETENTION IN LEAVES OF CONIFERS OF THE NORTHERN ROCKY MOUNTAINS

JOHNSON PARKER

Introduction

In the montane forests of northern Idaho there appears to be a variation in the distribution of certain species of conifers according to their ability to withstand drought. Ponderosa pine (*Pinus ponderosa* Dougl.) often occurs in pure stands on comparatively dry sites such as south-facing slopes, whereas Douglas fir (*Pseudotsuga taxifolia* [Lamb.] Britt.) commonly is present on more moist sites as, for instance, in ravines of south-facing slopes. Western arborvitae (*Thuja plicata* D. Don) is found on even more moist sites such as north-facing slopes and along the edges of streams.

Whatever the various synecological factors may be which affect distribution, certain distinct physiological factors play an important role in distribution. There is, for instance, evidence that depth of root penetration is a factor affecting survival of certain Rocky Mountain conifers (**7**). There is also evidence that structures other than roots are of importance as they affect drought resistance. When seedlings of ponderosa pine, Douglas fir, and arborvitae were grown in phytometers, the pines withstood a soil-drought period of 6 days with only slight mortality, while Douglas fir suffered a much higher mortality, and all arborvitae seedlings were killed (**3**). This suggests that there might be a difference in ability of the shoots to withstand drought when the roots are unable to absorb enough water to compensate for losses from the tops. If the drought resistance is in part

a property of the leaves, then it becomes a question as to whether the leaves of drought-resistant species retain more moisture than those of other species or whether leaves of drought-resistant species are only better able to recover from a low content of leaf moisture. The following experiments were carried out for the purpose of attempting to answer this question.

Material and methods

Branches were cut from trees of a number of different coniferous species which were growing in a single habitat. These species included ponderosa pine, Douglas fir, and arborvitae, as well as western white pine (*Pinus monticola* Dougl.), grand fir (*Abies grandis* Lindl.), and Engelmann spruce (*Picea engelmanni* [Parry] Engelm.). In the laboratory the leaves were plucked from the branches and separated into three or four groups, depending on the experiment, with about fifteen needles per group for each species. In the case of arborvitae, branches were cut so that about 2 inches of a twig were included with leaves attached. All leaves were placed in open Petri dishes and left on the laboratory table. Temperature was maintained at 25.5° C. and relative humidity at about 40%. All replications for each species were weighed daily on a balance accurate to tenths of milligrams. At the end of each test interval leaves were placed in an oven at 105° C. for exactly 48 hours and the dry weight recorded. Moisture content was then calculated as a percentage by dividing the oven-dry weight into the difference between leaf weight during the experiment and oven-dry weight.

The groups of leaves for tests 1 and 3 (table 1) were obtained at 3,000 feet elevation from the south side of Moscow Mountain and the group for test 2 at 4,800 feet elevation on the ridge adjoining the west side of the mountain. All samples shown for tests 1 and 2 were taken from their natural habitats in December, all others in January.

Another series of experiments was conducted to determine the comparative moisture loss from leaves of different species when the leaves were still attached to the branches. Leaves were sampled each day from branches which had been placed upright in the laboratory. They were then weighed, oven-dried, and reweighed. During this period measurements of leaf thickness were made with a screw-type micrometer. Sections were also made of leaves from time to time in order to observe changes in the various tissues.

After the drying period of from 2 to 12 days, an attempt was made to rehydrate the leaves and bring them back to a normal condition. Plasmolytic determinations proved to be indefinite, and the question arises in this connection as to whether the normal appearance of the protoplasm is an indication of viability. Measurement of CO_2 release by the leaves might be a measure of viability if there were no microörganisms present, but the amount of CO_2 given off is not a measure of the energy released nor does it give any indication as to what changes are occurring in the leaf (**2**).

One other test for relative viability of leaves suggested itself from work done in seed-testing with 2,3,5-triphenyltetrazolium chloride (**8**). ROBERTS (**11**) has shown that this salt is reduced in a number of tissues of vascular plants, but whether the activity of the enzymes involved is a real indication of viability remains a question to be answered definitely. The presence of active enzyme systems in seeds does not necessarily indi-

cate that they are viable, but the absence of active dehydrogenases affecting the tetrazolium compound is an indication of their lack of germinability (6). A positive tetrazolium test in leaves of conifers was found to be associated with the green color which is characteristic of healthy conifer leaves, as well as with a certain amount of flexibility and with a lack of the terpene odor characteristic of dead conifer leaves. On the other hand, leaves that have turned a dull green, have become brittle, or produce the dead-leaf odor will not show the tetrazolium test when rehydrated. The leaves will continue to show the test after a period of 3 weeks when cut off the branches, kept in moist air, and illuminated with an incandescent light at about 400 foot-candles. It thus seemed possible that some measure of viability was indicated by this test.

After 2 days of drying one group of leaves for each species was placed in distilled water at pH 5.5 for 1 day. Leaves were kept in contact with a free water surface on one side and with air on the other. This system of rehydration seemed to be as effective as keeping the leaves submerged in continuously aerated water. When the leaves had been rehydrated in 1 day's time, they were blotted, weighed, and sampled for the tetrazolium test. For this test leaves were cut into lengths 4 mm. long and placed in the depression of a hanging-drop microscope

TABLE 1

MOISTURE CONTENT OF DETACHED LEAVES AS PERCENTAGE OF DRY WEIGHT (FIRST NUMBER* IN EACH COLUMN) AND PERCENTAGE OF LEAVES SHOWING POSITIVE TETRAZOLIUM TEST (SECOND NUMBER*) AFTER BEING HYDRATED

Test 1	2 days		3 days		4 days	
Ponderosa pine	111	100	93	100	81	100
Douglas fir	118	100	73	100	68	100
Arborvitae	78	100	17	100	12	0

Test 2	4 days		5 days		6 days		12 days	
Douglas fir	103	100	109	100	105	100	9	0
Ponderosa pine	98	100	101	100	79	100	58	100
Arborvitae	73	100	28	0	45	100	10	0
White pine	62	66	95	100	20	0	9	0
Grand fir	62	66	42	33	26	0	9	0
Engelmann spruce	37	33	43	44	29	33	27	0

Test 3	4 days		7 days		9 days	
Ponderosa pine	95	100	62	90	10	0
Douglas fir	91	100	39	0	9	0
Grand fir	51	33	11	0	10	0
Arborvitae	43	100	8	0	8	0

* Each number represents separate set of leaves from different trees.

slide. The depression was filled with a 1% tetrazolium solution and covered with an ordinary slide to exclude air. The samples were then placed in the dark for 12 hours and observed for the appearance of the red color in the leaves which is the indication of a positive test. At various intervals thereafter leaves were removed from the drying dishes, rehydrated, and tested with tetrazolium solution.

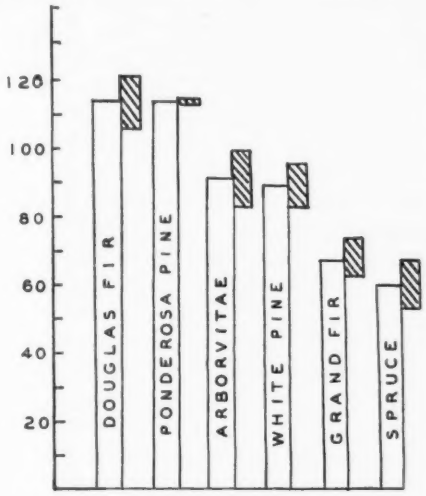

FIG. 1.—Moisture content of cut leaves shown by height of bars as percentage of dry weight after 3 days of drying. Scale in percentage. Mean for each species lies within distance shown by height of cross-hatched area with probability of 0.95.

Results

When leaves of various coniferous species were removed from the branches and dried in the laboratory, those of Douglas fir and ponderosa pine generally retained a higher moisture content than did those of arborvitae, white pine, grand fir, and Engelmann spruce. Figure 1 shows the combined results of three separate experiments in which leaves were brought from various habitats at 4800 and 3000 feet elevation. With a probability of 0.95 the mean for a species lies within the area indicated by the cross-hatched bar. This shows that Douglas fir and ponderosa pine were not significantly different in retention of leaf moisture and that arborvitae and white pine were not significantly different. Likewise, spruce and grand fir did not show significant differences. However, these three groups were significantly different from one another after 3 days of drying. Rates of drying are indicated by results shown in table 1.

When leaves were left attached to branches brought into the laboratory, there was a notable difference in the rates of moisture loss from those of the previously described experiments. After 6 days only Douglas fir and ponderosa pine showed significant differences, but after 12 days ponderosa pine was significantly different from the other three species with the usual probability of 0.95.

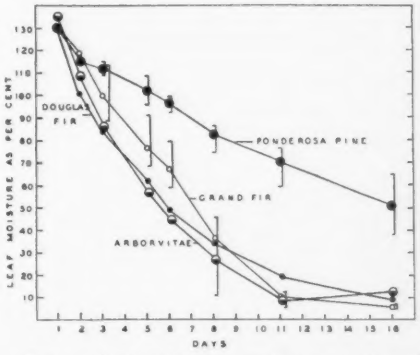

FIG. 2.—Leaf-moisture content as percentage of dry weight in leaves attached to cut branches in laboratory. Time scale indicates days since time of picking from trees. Vertical lines indicate spread of original data.

This difference between ponderosa pine and the other species is shown in figure 2 in which the dispersion of the data is given for ponderosa pine and grand fir.

At the same time that the experiment

with the attached leaves was begun a few leaves were plucked from each branch and dried separately on the table as in other experiments. Results were similar to those shown in figure 1. Ponderosa pine and Douglas fir withheld more moisture than the other species, and grand fir had the lowest moisture content after 2 days until 9 days, when all leaves of each species had reached a level of 10% moisture content. The only significant differences were shown between grand fir and ponderosa pine in this particular experiment.

A repetition of the experiment with attached leaves showed essentially the same results as before, although different trees were sampled from the same drainage on Moscow Mountain. The moisture loss in the first day is often an indication of the trend for the next 2 weeks. It is possible that the stomates close within 1–2 hours after cutting and bringing into the laboratory and that subsequent transpiration is largely cuticular.

Micrometer measurements showed a marked decrease in leaf thickness in the first few days of the experiment with attached leaves. This amounted to about 50μ per day at the rate of drying shown in figure 2. Ponderosa pine underwent the largest decrease in thickness, although this took place over a longer period of time than in the other species. Sections of leaves in paraffin revealed that much of the shrinkage in ponderosa pine needles is accounted for by a decrease in volume of cells of the transfusion tissue between the vascular bundles and the endodermis. There is comparatively little transfusion tissue in Douglas fir, grand fir, and red cedar leaves, and most of the shrinkage is accounted for by decrease in size of chlorenchyma cells. In leaves of grand fir and Douglas fir there may be a lateral curling of the leaves, as when seen in cross section, so that micrometer measurements are not always exact.

Discussion

It has generally been supposed that many relatively mesophytic vascular plants are able to recover from a drought period because they are able to withstand a low content of leaf moisture and not because of the amount of moisture retained in the leaves (**9**). This line of thought is supported by much work. For instance, SCHRÖDER (**12**) found that the moisture content of coniferous leaves could fall lower than that of leaves of broad-leaved species of trees without injury. NEGER and FUCHS (**10**) reported that when the moisture content of certain abscising conifer leaves fell to 20–30% on the basis of fresh weight, the leaves fell off the twigs. CHU (**1**) also concluded that there was a fairly definite "lethal borderline" moisture content for certain conifers and broad-leaved species and that it varied for different species. CHU, however, qualified his conclusions with the statement that the speed of drying and remoistening made some difference in the results. This qualification appears to reflect the ideas of ILJIN (**5**), who cast some doubt on the establishment of a definite lethal border-line moisture content because of the great variations in lethal effect from different treatments. ILJIN stated that a leaf cell that is repeatedly plasmolyzed and deplasmolyzed may die more readily than a leaf cell that is much more severely plasmolyzed and then only once rehydrated.

The idea that many comparatively xerophytic plants retain more moisture in their leaves than other plants has been frequently discarded in favor of MAXIMOV's broad conclusions. SHIELDS (**14**) discussed a number of xeromorphic structures in semidesert plants but appeared to favor MAXIMOV's point of view in re-

gard to water retention. This is shared by many plant physiologists. On the other hand, WEAVER and CLEMENTS (15) stated that although xerophytes may lose even more moisture than mesophytes when water is readily available to the roots, a different situation may occur when leaf moisture content is reduced to a low level and the stomates close. They pointed out that at this time cuticular transpiration is important and may be considerable with mesophytes but may be reduced to almost nothing in some xerophytes. Although such xerophytes are in a state of permanent wilting, their tissues may remain alive a long time.

The same sort of situation described by WEAVER and CLEMENTS may occur in many coniferous species. It was suggested by HUBER (4) that the drought resistance of certain pines and oaks might be explained by a greater reduction of water loss during time of drought than in the more mesophytic spruces and beeches. Although this point of view is often discredited, SCHOPMEYER (13) produced evidence that the more drought-resistant shortleaf pine (*Pinus echinata* Mill.) did retain more moisture in its leaves during a drought than did loblolly pine (*P. taeda* L.), a more mesic species. The results of the present paper also show that leaves of given species of Rocky Mountain conifers retain different amounts of water after their normal moisture supply is cut off and that this characteristic is associated with the natural occurrence of the species on sites differing in moisture availability.

It is possible that the apparent disagreement on the subject of leaf-moisture content and a "lethal level" is to be explained by the fact that the situation in many conifers and perhaps also in many semidesert plants is quite different from that in herbaceous plants and in many woody broad-leaved species. Much more work of this nature needs to be done before extensive generalizations can be made for the higher vascular plants.

Summary

1. Leaves of ponderosa pine (*Pinus ponderosa*) retained more moisture when attached to cut branches than did those of Douglas fir (*Pseudotsuga taxifolia*), western white pine (*Pinus monticola*), arborvitae (*Thuja plicata*), grand fir (*Abies grandis*), or Engelmann spruce (*Picea engelmanni*).

2. Leaves of Douglas fir and ponderosa pine retained a higher moisture content on the average than did those of arborvitae, white pine, Engelmann spruce, or grand fir when the leaves were removed from the branches and allowed to dry in the laboratory.

3. Cut leaves of arborvitae and white pine showed no significant differences in moisture retained, but cut leaves of both species retained more moisture than did leaves of grand fir or spruce.

4. If the tetrazolium test is a test of relative viability, it did not indicate any results to substantiate the claim that the more drought-resistant coniferous species are better able to recover from a low content of leaf moisture than are those less drought-resistant. In general, a loss of leaf moisture below 50% on the basis of dry weight, and remaining below this level for 3 days, resulted in no tetrazolium test when rehydrated.

5. Leaf shrinkage was closely associated with water loss and appeared to be most marked in the case of ponderosa pine, although this took place over a longer period of time than with Douglas fir, grand fir, or arborvitae.

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PECTOLYTIC ENZYME ACTIVITY IN VARIOUS PARTS OF THE CUCUMBER PLANT AND FRUIT[1]

THOMAS A. BELL[2]

Introduction

Each year the pickle industry suffers considerable economic loss caused by an uncontrollable type of spoilage known as softening of cucumber salt-stock. Recently, BELL *et al.* (1) identified a causative agent in commercial pickle brines to be a pectin-splitting enzyme, related to polygalacturonase, and gave a method for measuring its activity in the brines. Before the pickle industry can expect to have reliable methods for controlling or preventing spoilage caused by enzymatic softening, the source of the pectin-splitting enzyme must be investigated.

The present study deals principally with the extent of pectolytic enzyme activity in the seeds, flowers, petioles, stems, and fruit of several varieties and sizes of pickling cucumbers (*Cucumis sativus* L.). No attempt was made fully to characterize the pectolytic enzyme. The work indicated that certain parts of the plant and the mature whole fruit may be the source of the softening enzyme rather than microbial activity as heretofore supposed.

As ripe tomatoes (*Lycopersicon escu-*

[1] This study was carried out under a co-operative project with the Department of Horticulture of the North Carolina Agricultural Experiment Station, Raleigh, North Carolina.

[2] Chemist, Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.

lentum Mill.) are known to contain a pectolytic enzyme, the study included measurements on Marglobe variety as a check on the viscosity method and also to determine whether any differences occurred during development of the enzyme system in fruits of the two plants.

General methods of commercial salting of cucumbers for pickles have been described by ETCHELLS and JONES (2) and by others (3, 19) and will not be given in this report.

Nomenclature

The nomenclature of the pectolytic enzymes which catalyze the hydrolysis of the glycosidic bonds of pectin and also cause a reduction in viscosity of pectin or pectic acid has been reviewed recently (12, 15, 18). These enzymes are referred to as pectinase and polygalacturonase. More recently (13), an enzyme slightly different from polygalacturonase has been reported in ripe tomatoes and named pectic acid-depolymerase.

In the present study the general term "pectolytic enzyme" is used. Although the enzyme is similar to polygalacturonase, its characteristics are not yet fully known. When cucumber salt-stock becomes soft, the glycosidic hydrolysis of pectin or pectic acid to galacturonic acid would not necessarily have to be complete. Only a slow and partial depolymerization of the pectin molecule would be required. The work of FABIAN and JOHNSON (4) indicates that this is true because they found the total pectin content of both mushy and firm salt-stock pickles to be the same when measured as calcium pectate. The glycosidic enzyme system found in the brines from soft salt-stock, or in the extracts of the cucumber and tomato tissues reported here, may be only a depolymerase and, if so, would not hydrolyze pectic acid to galacturonic acid.

Review of literature

There are numerous sources of the pectolytic enzymes. A recent article by MATUS (12) lists sixty-six specific sources for pectinase, including bacteria, actinomycetes, molds, fungi, higher plants, and animals. It is doubtful if all sources above are correct, since methods for testing the enzyme vary with different workers, and much of the work was done without the newer knowledge of pectin and pectic enzymes. As an example, the pectic enzyme from *B. mesentericus fuscus*[3] claimed by FABIAN and JOHNSON (4) to be responsible for softening of cucumber salt-stock could not be the polygalacturonase-like enzyme system found in soft-cucumber brines or in the commercial pectinols (1), because of the outstanding differences in chemical properties (e.g., pH, acidity, and salt tolerance of the enzyme systems).

The extensive studies (5, 7, 8, 10, 16) with the polygalacturonase enzyme system have been based on molds as the source. Because the present study deals with a pectolytic enzyme active in varieties of pickling cucumbers and in tomatoes, sources from higher plants reported in the literature are of greater interest.

In 1921 PATON (17) demonstrated that Easter lily pollen contained pectinase. In summarizing, he pointed out the following: "Histological examination shows that in most instances pollen tubes make their way between the walls of adjacent cells rather than penetrating them. We should expect therefore to find most frequently not a cytase- or cellulose-digesting enzyme, but rather a pectinase ca-

[3] BERGEY'S *Manual of Determinative Bacteriology* (6th ed., 1948) lists *B. mesentericus fuscus* as a synonym for *B. subtilis*.

pable of digesting the pectin of the inner lamella."

In 1938 KERTESZ (9) found polygalacturonase activity in ripe tomatoes but stated that it apparently was absent in the green tomato. The method he used to indicate glycosidic hydrolysis was based on a decrease in alcohol precipitate of a pectin solution when acted upon by the tomato extract. He was not satisfied with the change in viscosity of a pectin solution for measuring polygalacturonase activity. MACDONNELL *et al.* (11) found polygalacturonase activity in ripe tomatoes and observed an increase in reducing groups liberated from pectic acid as determined by the WILLSTÄTTER-SCHUDEL hypoiodite method. They observed that tomato extract was twice as active at pH 4.0 as compared with its activity at pH 5.6. MOTTERN and HILLS (14) also observed polygalacturonase activity in tomato extract and found a decrease in viscosity of 0.9–1.2% per hour when 1.0 ml. was added to 200 ml. of a 1% pectin solution at pH 4.0 and at 30° C. Recently, MCCOLLOCH and KERTESZ (13) reported a polygalacturonase-like enzyme in ripe tomatoes and named it pectic acid-depolymerase. They considered this enzyme to be different from polygalacturonase of mold origin because depolymerization of pectic acid was not complete to galacturonic acid and the tomato enzyme was more heat-resistant.

Material and methods

EXTRACTION.—Freshly picked cucumber and tomato plant tissues were washed with cool tap water, graded for well-developed healthy fruits, and assorted to respective sizes. Four varieties of cucumber—Producer, Model, National, and Packer—and the Marglobe variety of tomato were represented.

The cucumber and tomato fruits were then frozen in sealed cans and held at −10° C. Later, they were partially thawed and were ground with small amounts of distilled water in a Waring Blendor with 2% sodium chloride. The pH values of the blended materials were then determined, and those below pH 6.0 were adjusted to this value with 1*N* sodium hydroxide. The juices were extracted through heavy, fruit-press cloths, made to known volume, and stored under toluene at 5° C. The leaves, flowers, petioles, stems, and dry seeds[4] of the cucumbers and the embryo and flower of the tomato were blended directly without freezing. The extraction procedure was similar to that used by HILLS and MOTTERN (6) for pectinesterase of tomatoes.

PECTOLYTIC ENZYME MEASUREMENTS.—The method described by BELL *et al.* (1) was used, which, briefly, is as follows: Two 25-ml. amounts of approximately 3% pectin[5] solution, having a relative viscosity of about 30 and buffered at pH 4.0 with sodium citrate–citric acid, were placed in 50-ml. Erlenmeyer flasks. Five milliliters of the extracted juice were placed in one flask, and 5 ml. of the same extract which had been heated to 80° C. in a water bath for 10 minutes to inactivate the enzyme were placed in the other. After an incubation period of 6 days at 30° C., the dropping time from the pipette and apparatus previously described (1) was determined. The measurement of activity was expressed as percentage loss in viscosity of the unheated extract over that of the control. Values so obtained are not a linear quan-

[4] Kindly supplied by Mr. FRANK B. FAUST, Manager, Associated Seed Growers, Atlanta, Georgia.

[5] Pectin NF was used and was obtained from California Fruit Growers Exchange, Ontario, California. The mention of trade-name products does not imply that they are indorsed or recommended by the Department of Agriculture over other similar products not mentioned.

titative measurement of enzyme activity, as demonstrated previously (1).

Where gel formation interfered with the viscosity test, the extracted juices were treated to destroy the pectinesterase prior to the enzyme determinations. This was done by incubating the extract at pH 3.0 for 24 hours at 40° C. These conditions used for destroying pectinesterase also cause some reduction in activity of the enzyme responsible for loss in viscosity of pectin. This was demonstrated by lower values for controls which were obtained upon treating non-gel samples.

Results and Discussion

CUCUMBERS.—From the results of measurements of pectolytic enzyme activity in the cucumber materials (table 1), it appears that the cucumber itself may be an important contributing factor to softening.

Glycosidic hydrolysis as indicated by percentage loss in viscosity of a pectin solution was demonstrated in many parts of the cucumber plant and fruit (table 1). Extracts from dry cucumber seeds were strongly positive; losses in viscosity of the pectin solution ranged from 44 to 72%. Extracts of leaves, petioles, and stems for the variety examined were all negative for enzyme activity. Staminate flowers and the pollinated pistillate flowers of two varieties, Producer and National, were strongly positive; but the unpollinated were negative. This would indicate that the pectolytic enzyme is produced by the staminate flowers and transmitted in the pollen grains by insects to the pistillate flowers.

Green, whole fruits were weak to negative in enzyme activity, whereas the ripe, whole fruits of the Producer, Model, and National varieties were strongly positive. Seeds in the yellow to orange-colored ripe fruits were more mature, and the enzyme activity most likely ties in with the seed development. This may offer an explanation for the soft-center type of spoilage found in large-sized salt-stock made from cucumbers approaching maturity. It is of added interest that the

TABLE 1

PECTOLYTIC ENZYME ACTIVITY AND PH OF EXTRACTED CUCUMBER TISSUE

Cucumber tissue tested	pH	% pectolytic enzyme activity as loss in viscosity*
Seeds (10% extract):		
Producer var.	6.5	44
Model var.	6.2	68
National var.	6.4	50
Packer var.	6.4	72
Leaves, Producer var.†	7.0	0
Petioles, Producer var.	6.4	0
Stems, Producer var.	6.0	0
Flowers (approx. 2% extract):		
Staminate		
Producer var.	6.3	91
National var.		88
Pistillate and embryo		
Unpollinated		
Producer var.	7.40	0
National var.	7.40	0
Pollinated		
Producer var.	7.20	89
National var.	7.00	84
Green whole fruit:†		
Producer var.		
Range in sizes (gm.)		
5–10	6.1	12
10–25	5.9	9
25–50	5.8	9
50–75	5.7	7
75–100	5.8	0
100–150	5.7	0
150–200	5.8	1
200–300	5.2	22
Ripe whole fruit:		
Producer var.	4.4	56
Model var.	4.5	43
National var.	4.4	53

* Degree of pectolytic activity: 0–3%, negative; 4–10%, weak; 11–30%, moderate; above 30%, strong. The percentage loss in viscosity values is not a linear quantitative measurement.

† The extracted juice with pectin solution gave a gel caused by the enzyme pectinesterase. To avoid this, extracted juice was adjusted to pH 3.0, incubated at 40° C. for 24 hours, and then tested on pectin solution for enzyme activity.

pH value of the extracted fruit decreased as the fruit developed—the value for the small (green) fruit was 6.1, whereas for the ripe it was 4.4. Pectolytic activity was found in the extracts of mature fruits where the pH of 4.4 is near the optimum for polygalacturonase activity (**12, 18**) and also for the softening enzyme (**1**) of commercial brines.

These findings put an altogether new light on the problem of softening of commercial cucumber brine stock. Heretofore, microbial activity in the brine has been assumed to be the sole enzyme source.

TOMATOES.—Pectolytic enzyme activity was absent in the embryos with flowers, and in six stages of development of the green fruit of the Marglobe tomato starting with fruit of less than 10 gm. in weight to those of 150 gm. Fruits in the three stages of ripeness, as indicated by red skin color of one-third, two-thirds, and all red, were moderately to strongly positive.

This work confirms other reports (**9, 11, 13, 14**) that ripe tomatoes contain a polygalacturonase-like enzyme. However, the absence of this enzyme in the early developmental stages and in the green fruit has not previously been clearly demonstrated. Sizable quantities of green tomatoes are brined yearly by the pickle industry for use as dills, relishes, cut pickle, and pasteurized products. The absence of the softening enzyme in the green fruit may, in part, account for the lack of difficulty experienced by industry in brining this commodity.

Summary

1. The cucumber plant and fruit (*Cucumis sativus*) have been found to be a source of a pectolytic enzyme as measured by a loss in viscosity of a pectin solution.

2. The enzyme of the cucumber was strongly active in the seeds, staminate and pollinated pistillate flowers, and ripe fruit but was not found in the unpollinated flowers, leaves, petioles, and stems.

3. Enzyme activity was weak to negative in the eight stages of cucumber development of immature fruit.

4. Pectolytic enzyme activity was absent in the green tomato (*Lycopersicon esculentum*) including the embryo with flowers and six stages of green-fruit development.

5. High activity of the enzyme was found in the red ripe tomato fruit.

The author is indebted to Dr. JOHN L. ETCHELLS of this laboratory for his cooperation, advice, and many suggestions, including the use of cucumber pollen as a source of the pectin-splitting enzyme. Acknowledgement is made to the following members of the Horticulture Department, North Carolina State College, for their advice and assistance and for supplying the cucumbers and tomatoes: Professor M. E. GARDNER, Head of the Department, Dr. IVAN D. JONES, Mr. ALBERT A. BANADYGA, Mrs. MARY GUY, and Miss ELEANOR GIBBS. The author also wishes to acknowledge the help of the CHAS. F. CATES Company of Faison, North Carolina, and the Mount Olive Pickle Company, Mount Olive, North Carolina, for use of their facilities.

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OLEORESIN COMPOSITION AS A DETERMINANT OF PINE HYBRIDITY

BRUCE ZOBEL

Introduction

Recently, a study was made of the natural hybrid between *Pinus coulteri* Lamb. and *Pinus jeffreyi* Grev. and Balf., two species of pines found growing in association only in central and southern California. Fifteen characters were used to differentiate the two parental species, including those based on the morphological and anatomical features of the cone, foliage, and seed. In addition, the characters of the oleoresin collected from each tree were used. The techniques and results of analysis of this oleoresin will be of interest to botanists, geneticists, and foresters.

It has been known for some time that the various species of pines have oleoresins with different characters, usually specific in nature; this was reported early for the American pines by SCHORGER (**9**). MIROV (**6**) pointed out the value of the use of oleoresins in a taxonomic study when he stated that each pine species as a whole has a specific turpentine by which the pine can be easily identified.

In addition to normal taxonomic studies, the use of this biochemical character is of value in the analysis of some pine hybrids, especially those similar to the Coulter–Jeffrey pine hybrid whose parental species have vastly different oleoresin compositions. The type of oleoresin to be expected in a hybrid is not definitely established. However, the oleoresin of a few Jeffrey–ponderosa pine hybrids was intermediate (4), and the recently completed analysis of some forty Coulter–Jeffrey hybrids (and one hundred and ten parental trees) points to a definite intermediacy between the parental species in all the oleoresin characters studied. In addition, a plantation of F_2 Jeffrey–ponderosa pine hybrids has been established at Berkeley, California, by the Institute of Forest Genetics, Placerville, California. MIROV stated that preliminary tests of the aldehyde in the oleoresin of ninety of these trees indicated that this chemical character did not follow a simple Mendelian segregation. Thus, intermediacy exhibited by oleoresin characters should be as valuable in the analysis of a hybrid as intermediacy in the morphological and anatomical characters.

Material and methods

Collection of the oleoresin of the Coulter–Jeffrey pine hybrid was made using a modification of standard turpentining procedures (11). In past studies involving this biochemical characteristic, it was felt necessary to collect a large volume of material in order to make an analysis. This required setting out the cups and leaving them for a considerable period of time until the trees had "bled" sufficiently, often requiring several return visits to each tree. However, in this analysis of the Coulter–Jeffrey pine hybrid, a "micro" method was worked out with the aid of Dr. N. T. MIROV of the Institute of Forest Genetics, in which it was determined that one-half test tube of the oleoresin would be sufficient for later analysis, thus making possible collection of the oleoresin in a small container such as a test tube. This usually required only 1 or 2 days of "bleeding," enabling the sampling of a large number of trees in one brief visit to any area studied. When collection was complete, the test tubes were corked and then were placed in a low-temperature room as soon as possible. In the laboratory the oleoresin was separated into its steam-volatile and nonvolatile fractions by the standard steam distillation method (3). After several trials it became evident that satisfactory results were obtained when 20–30 gm. of the oleoresin were distilled in 250 cc. of water in a large flask; this yielded approximately 5–7 cc. of oil, a quantity sufficient for all later analyses.

The characteristics for which analyses were made were limited entirely to the steam-volatile portion of the oleoresin, since very little is known at the present time about the nonvolatile portion, or rosin. No attempt was made to undertake a complete analysis of the volatile oil; consequently, further fractionations and specific chemical determinations were not attempted. Throughout this study all tests were restricted to the simplest and most practical types, so that oleoresin analysis could be a tool that would not make necessary complex equipment or an extensive knowledge of chemistry.

At first the volatile oil was subjected to the usual tests whereby the optical rotation, specific gravity, and index of refraction were determined. These are simple measurements which are determined for most analyses of organic materials such as the volatile oils. Such measurements

are very valuable in a hybrid study; however, the determination of the optical rotation requires a polarimeter, and the determination of the index of refraction requires a refractometer, instruments not usually found in a small laboratory. For this reason an attempt was made to devise a method requiring even less elaborate equipment. With the aid of Dr. ARTHUR ANDERSON of the Forest Products Laboratory of the University of California, a method of analysis was devised that will here be called the sulfuric (acid) nonsoluble test; this is a modification of the standard fuming sulfuric acid method (2) and suggested for use here by Dr. N. T. MIROV. This test is based upon the fact that the unsaturated hydrocarbons and oxygenated organic compounds react with concentrated sulfuric acid to form soluble derivatives, while saturated hydrocarbons generally are found to be insoluble in this same concentrated sulfuric acid. It enabled an easy estimate of the percentage of material which was not soluble in concentrated sulfuric acid while using only 1 cc. of the volatile oil.

The volatile oil was tested for solubility in a Babcock milk-test bottle about two-thirds full of concentrated sulfuric acid. (This bottle has a graduated neck, but its graduations are not in cubic centimeters, so it must be calibrated before using.) To the acid was added exactly 1 cc. of the oil to be tested, and the mixture was shaken until it was thoroughly blended and until heating had ceased. Then the bottle was filled with acid until it projected up into its neck. This mixture was left standing for a specified time (24 hours were used in this study), and the percentage of oil that was nonsoluble could be read directly from the volume of the colorless immiscible liquid which rose in the bottle's neck.

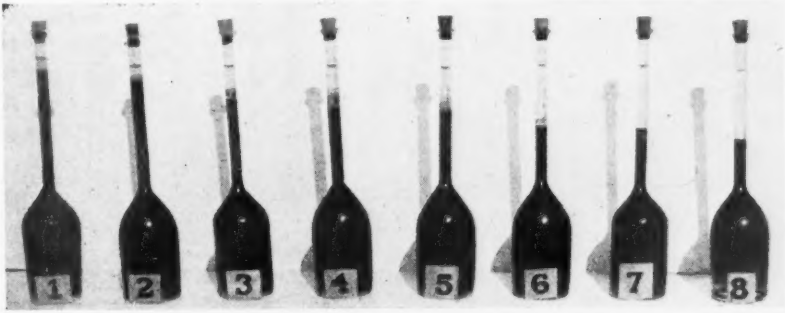

FIG. 1.—Series of Babcock milk-test bottles showing percentage of nonsolubles in volatile oil of oleoresin. Note that all bottles were filled to same level, as shown by menisci just below corks. Nonsolubles compose the clear liquid in bottle neck. Bottle on left contains oleoresin of typical Coulter pine; one on right contains oleoresin of typical Jeffrey pine.

Results and discussion

Some of the results obtained by the foregoing test are shown in figure 1, where the parental species are represented by bottle 1 (Coulter pine) and bottle 8 (Jeffrey pine). Bottles 2 through 6 represent trees of varying degrees of hybridity.

This test was particularly applicable to the Coulter–Jeffrey pine hybrid because Jeffrey pine contains 95% heptane —a saturated hydrocarbon—while Coul-

ter pine contains only 5% heptane and 10% undecane—another saturated hydrocarbon (**7, 8**). Therefore, it would be expected that approximately 95% of the volatile oil of Jeffrey pine should remain unaffected by sulfuric acid, while only about 15% of the volatile oil of Coulter pine should be unaffected. This proved to be the case, for it was found that on the average 93% of the oil of Jeffrey pine was nonsoluble, while Coulter pine showed 15% nonsoluble material. The hybrids all showed an intermediate percentage of nonsoluble material, as illustrated in figure 1. Bottle 5 shows the results obtained from a first generation cross, or F_1, the others being backcrosses to the parental species or segregates from the crossing of two hybrids. This test was very satisfactory in the analysis of the Coulter–Jeffrey pine hybrid, but its applicability to other pine hybrids must be determined.

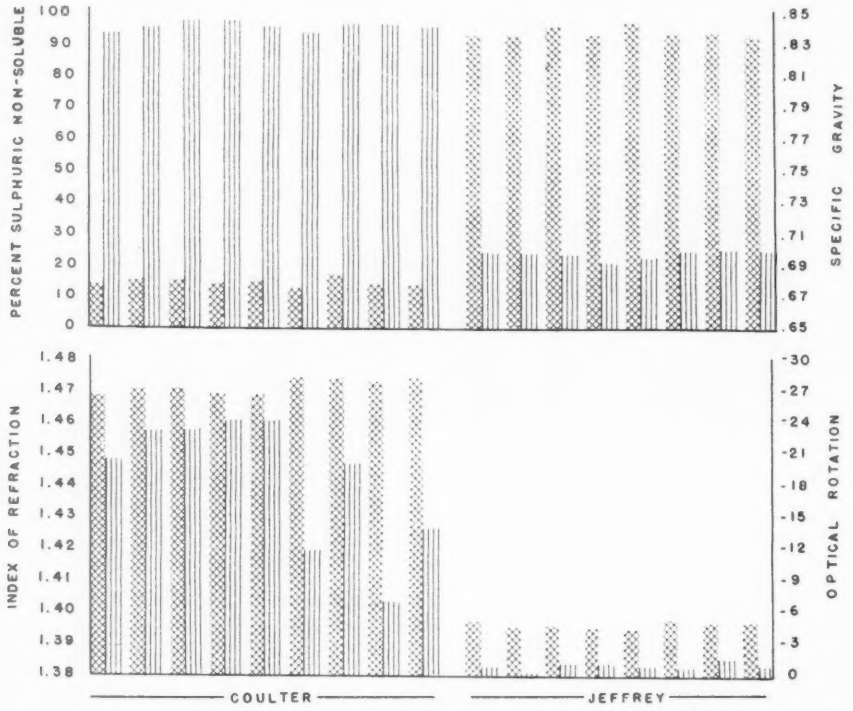

FIG. 2.—Constancy of four oleoresin characters shown by samples from populations from all parts of species range. Crosshatched areas represent indexes of refraction and sulfuric acid nonsolubles; areas of vertical lines represent optical rotation (laevorotatory) and specific gravities. Most northerly regions for each species are plotted on extreme left, proceeding more southerly as read to right.

One of the outstanding results from this study of oleoresins was the constancy of the results obtained throughout the ranges of the parental species, as illustrated by the averages for the populations in figure 2. Such constancy in the parental species is extremely important in the study of a hybrid, for then any variation present in a suspected hybrid

will be of considerable significance. The absence of variation among individual trees and among the averages for different populations was true for all measurements made except optical rotation; here, there was much variation among individual trees (especially for Coulter pine) and some variation in the averages for different geographical regions, with a tendency for this variation to follow a geographic pattern.

The results obtained for some individual hybrid trees, along with the averages for the parental species, are shown in figure 3. Note that a great many of the hybrids appear to be strong backcrosses to the Coulter pine parent, a result which is substantiated by the other characters analyzed. There is a very close similarity in the intermediacy shown by the oleoresin characters and the intermediacy determined by correlating the fifteen characters based on foliage, cone, seed, and oleoresin, determined by the use of ANDERSON's hybrid index (1, 10). In applying this index, the oleoresin characters were given the same weighting as the other morphological and anatomical characters.

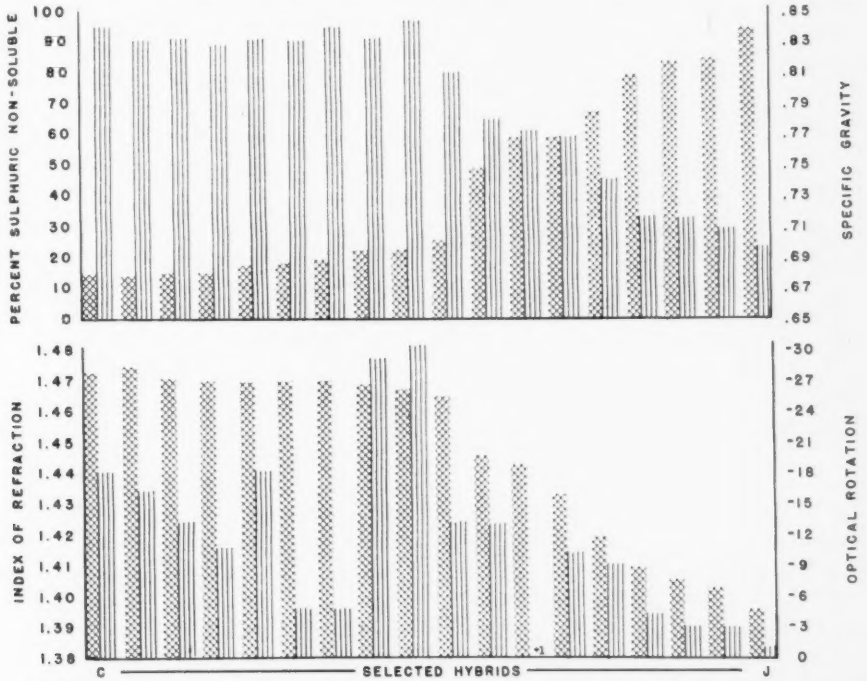

FIG. 3.—Intermediacy shown by individual hybrids for four oleoresin characters. Average for Coulter pine is plotted at left; average for Jeffrey pine at right. Crosshatched areas represent indexes of refraction and sulfuric acid nonsolubles; areas of vertical lines represent optical rotations (laevorotatory) and specific gravities.

Preliminary tests were made of the forty hybrids to determine if the four oleoresin characters were correlated. Results showed a remarkable degree of correlation among the specific gravity, the

index of refraction, and the percentage of sulfuric nonsolubles, with somewhat poorer correlation evident when the optical rotation was involved. This indicates that these characters are controlled by the same set of genes and that in a study of a natural hybrid such as the Coulter–Jeffrey pine hybrid, perhaps the measurement of only one oleoresin character would be sufficient. However, not enough hybrids were analyzed in this study to determine definitely if this is the situation.

Oleoresin analysis proved to be of considerable definitive value in the study of the Coulter–Jeffrey pine hybrid. In several cases the final classification as to the tree's hybridity was based largely on the character of its volatile oil. Also, analysis of the volatile oil enabled the detection of occasional unsuspected hybrids, thus contributing to a more complete and accurate survey of the hybrids. This was shown when studying an isolated group of trees growing on serpentine soil in the Sierra Nevada Mountains (Plumas County). The trees here were sampled as "pure" Jeffrey pines, although one tree was observed to have characters differing slightly from the others; however, these characters were still within the normal limit of variation of Jeffrey pine. When the oleoresin of this tree was analyzed, the characteristics of its volatile oil were very different from those for any Jeffrey pine; for example, the sulfuric acid nonsoluble test showed that only 20% of the oil was unaffected, whereas "pure" Jeffrey pine would have had over 90% unaffected. This, plus the results shown by the other oleoresin characters analyzed, enabled the postulation to be made that the tree was a Jeffrey–ponderosa pine hybrid. Similar situations were encountered for the Coulter–Jeffrey pine hybrid.

The importance of the application of oleoresin analysis in a study of a pine hybrid may be summarized by the following: (*a*) The measured results are objective, the characters being definitely measurable, thus allowing the use of statistical methods of analysis. (*b*) If the "micro" method is used, it is relatively rapid and no great amount of technical skill is required, either in the field collection of the oleoresin or in its analysis in the laboratory. (*c*) Oleoresin is a biochemical character, a valuable addition to the usual anatomical and morphological characters commonly employed in hybrid analysis; it is an approach to the often advocated, though seldom used, physiological study. (*d*) According to some advocates of the use of oleoresins, this biochemical character will not be so susceptible to environmental influences as most morphological characters, and therefore any change noted will have a greater significance. (*e*) It sometimes enables the detection of hybrids that otherwise might have been overlooked.

The usefulness of the oleoresin character depends on the development of more simple tests, and if more strictly field tests can be perfected, such as those proposed by MIROV (5), it should become one of the standard tools used in the study of the genus *Pinus*.

Summary

1. The use of oleoresin analysis (a biochemical character) is relatively new, but it is felt that it is of particular value in analyzing a pine hybrid as shown in a recent study of the natural hybrid between Coulter and Jeffrey pines. The field collection techniques are simple and rapid, and the laboratory analysis may be made without the necessity for complex laboratory equipment or an extensive knowledge of chemistry.

2. The volatile oil portion of the oleoresin is subjected to the standard tests whereby its specific gravity, index of refraction, and optical rotation are obtained. In addition, a simple method for obtaining an estimate of the degree of hybridity in the Coulter–Jeffrey pine hybrid is used, whereby the percentage of material in the volatile oil not dissolved by concentrated sulfuric acid is determined directly using only 1 cc. of the oil. These four characters appear to be closely correlated, necessitating the use of only one of them in analyzing the oleoresin of a hybrid.

3. The oleoresin characters are very constant throughout the parental species, thus making any variation found in the hybrid extremely significant. Occasionally the use of the oleoresin character enables the detection of backcrosses with all visible characters so similar to the parental species that ordinarily no separation would be made. In addition, it is a relatively rapid, objective method of analysis of a biochemical character, which, according to some investigators, is only slightly influenced by environmental variations.

The author wishes to express his appreciation to Dr. N. T. MIROV of the Institute of Forest Genetics, Placerville, California, and to Dr. A. ANDERSON and Dr. R. A. COCKRELL of the School of Forestry, University of California, for their guidance in the research and aid in preparation and revision of the manuscript.

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A RECORD OF SEQUOIA FROM THE JURASSIC OF MANCHURIA

SEIDO ENDÔ[1]

With an Introduction by

RALPH W. CHANEY

Introduction

Fossil specimens of *Sequoia* have excited the interest of paleobotanists and botanists for nearly a century, since HEER first recognized foliage and cones from the Oligocene of Switzerland. Its presence has been recorded in many lands, a distribution strikingly in contrast with the modern restriction of *Sequoia* to the western United States. Although many of these records, especially in the Arctics and in North America, are now known to have been based on specimens of *Metasequoia*, there is still ample evidence of the cosmopolitan occurrence of *Sequoia* at middle-northern latitudes during Cretaceous and Tertiary time.

No previous record of its presence is known in rocks older than the Cretaceous. In fact, few existing genera of woody plants seem to have appeared before the beginning of that period. So the discovery of *Sequoia* in beds of Jurassic age is of exceptional interest, especially since its first known area of occurrence is in Manchuria, far distant from the present home of its survivors. On the basis of the evidence now at hand, we may suggest that *Sequoia* had its origin in Asia and that it came into existence before either of its close relatives, *Metasequoia* and *Taxodium*. Such a suggestion can now be no more than tentative, since future discoveries may further extend the geographic and stratigraphic range of one or all of these genera of the Taxodiaceae.

The leafy shoot here described by SEIDO ENDÔ from Jurassic deposits in South Manchuria is so like the foliage of the living coast redwood, *S. sempervirens* Endlicher, as to indicate that it has changed but little during the last one hundred and twenty million years. The writer of this introductory statement wishes here to express his appreciation to Dr. ENDÔ for his discovery and description of this ancient *Sequoia*. It would be gratifying if he had had more material for study, including cones, but the student of past life must make use even of fragments. With his broad knowledge of fossil and living plants, Dr. ENDÔ has used this foliage fragment well, extending the history of *Sequoia* to an earlier frontier of earth history.

The fossil record

This report is based on a branchlet of a fossil *Sequoia* from the *Lycoptera* bed of Lingyuan, Jehol, in South Manchuria. It is probably the oldest known specimen of *Sequoia* in the world. Its description follows:

SEQUOIA JEHOLENSIS ENDÔ, N.SP.—Branchlet with one spray, about 15 cm. long and 1 cm. wide. Leaves of two types, scaly and ordinary; ordinary leaves with decurrent base, sessile, spirally arranged in two lines, having twisted base, linear or lance-shaped, and bluntly mucronate

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FIG. 1.—*Sequoia jeholensis* Endô, n.sp. ×1. Locality: *Lycoptera*-bed of Lingyuan, Jehol, South Manchuria. Holotype.

FIG. 2.—Same specimen as in figure 1 enlarged ×1.55 to show details.

at apex, about 10 mm. long and 1 mm. wide, upper surface slightly furrowed along the midrib; scaly leaves, which occur in growth of early spring and at base of lateral shoot, spirally arranged, triangular shaped with decurrent base, midrib distinct. IGPS coll. cat. no. 60980.

REMARKS.—The present material shows close resemblance to the branchlets of the existing *S. sempervirens* Endlicher, of which it may be a direct ancestor.

According to F. TAKAI (3), the fossil-bearing bed is Upper or Middle Jurassic in age from the evidence afforded by the lycopterid fishes; thus the present record is the oldest one known with certainty. The most ancient *Sequoia* remains hitherto reported are from the Upper Jurassic of the Boulogne district. This species, originally described as *S. problematica* Fliche and Zeiller (2), is founded on a small elliptical cone whose relationship to *Sequoia* has not been demonstrated.

Sequoia remains are very abundant in Cretaceous rocks. Among them, *S. heterophylla* Velenovsky (4) from the Lower Cretaceous of Bohemia shows little resemblance to the present material in the feature of the foliated branchlet. *S. fairbanksi* Fontaine from the Lower Cretaceous of California (5) represents foliage of a type unlike any known *Sequoia*. Most of the foliage specimens of this genus from the Upper Cretaceous of the northern hemisphere are of the *S. reichenbachi* (Geinitz) Heer type, with the scaly needles characteristic of *Sequoiadendron*. An Upper Cretaceous species, *S. dakotensis* Brown (1), from North Dakota is the oldest American *Sequoia* with distichous foliage.

It is expected that the discovery of *Sequoia jeholensis* will throw additional light on the history of the genus.

The writer wishes to express his sincere thanks to Professor R. W. CHANEY of the University of California for his valuable advice and suggestions during the present study.

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DISTRIBUTION OF WAXES IN GUAYULE[1]

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Introduction

The occurrence of a "waxlike substance" on the foliage of guayule (*Parthenium argentatum* Gray) has been reported by WALTER (3) in connection with a general investigation of guayule resin constituents. This waxy fraction was obtained by acetone extraction of the comminuted leaves and flower stems and constituted some 0.25% of the lush foliage, or about 3–4% of the resin (acetone extract). It was also obtainable in lower yields from acetone extracts of the whole shrub and from the resinous rubber. He reported the following properties for the recrystallized wax: 80.18% carbon; 13.25% hydrogen; saponification number 74.4; molecular weight (Rast) 597; melting point 76° C.

We have confirmed these findings and have sought practical methods for the isolation of this waxy fraction both from the other resin constituents and from the whole shrub. The wax was studied principally from the standpoint of its distribution in various parts of the plant with some attention to seasonal variation.

Analyses

Since 95% ethanol has a high temperature coefficient of solubility for the waxes, this solvent has proved generally useful for both extractions and recrystallizations. At reflux temperatures it has high solubility for the wax fraction, but at room temperature a very limited solubility for some of the more soluble cuts, and virtually none for a fraction (from leaves) purified by recrystallization three times each from benzene and acetone. The same purified fraction had the following weight percentage solubilities at 25° C.: acetone 0.00; glacial acetic acid 0.01; diethyl ether 0.10; benzene 0.39. This fraction had d_4^{76} 0.80 determined in an Ostwald pycnometer and d_4^{25} 0.963 based on the weight of a pressed pellet of known volume.

FIG. 1.—Appearance of guayule plants as taken from field.

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Other and more precise determinations of guayule wax solubility were not made, because saponification studies indicated that even the white recrystallized material, melting sharply at 76° C., is not a single compound but presumably a mixture of several.

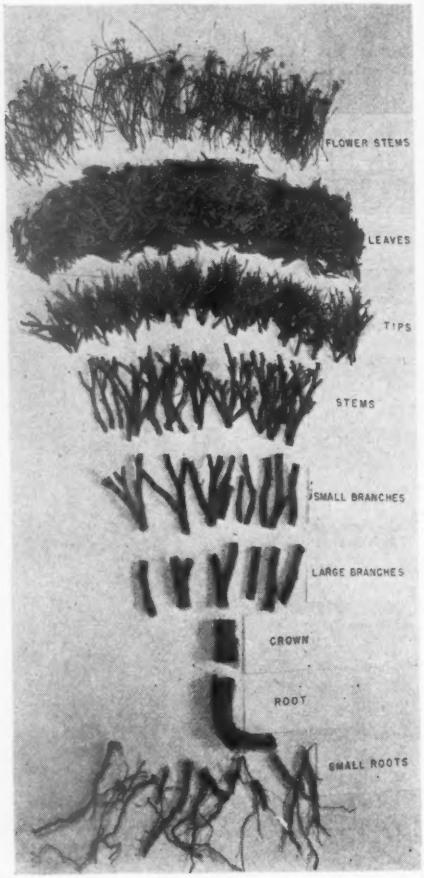

FIG. 2.—Segments into which plants were separated for analysis.

Although guayule wax can be extracted by refluxing the comminuted plant tissue with either acetone or 95% ethanol, the latter solvent is preferred for this work. The use of acetone causes the removal of a low-molecular-weight "rubber" fraction (1) which complicates purification of the waxy material. On standing or refrigeration the crude wax precipitates readily from dilute (< about 5% resin) ethanolic extracts from which it can be be filtered and recrystallized if desired. Recent studies in this laboratory have indicated that the principal contaminants responsible for the dark color of the crude wax are plant pigments, including chlorophyll and xanthophyll, which can be removed more or less effectively by percolation of dilute solutions through activated adsorbents.

A typical wax separation procedure and one which was used to estimate yields in conjunction with the wax distribution study was conducted in the following manner. Fifteen grams of finely cut shrub tissue were extracted for 6 hours with 150 ml. of 95% ethanol in a 250-ml. Soxhlet extractor. After the miscella had stood overnight, the precipitated wax was collected on a filter paper, washed with 100 ml. of 95% ethanol, dried in a vacuum oven, and the melting point determined. By this arbitrary procedure deviations in the percentage of wax recovered from replicates of the same shrub lots generally varied less than 5%.

A series of determinations was made on the percentage of wax in various parts of the guayule shrub, variety 593, by the procedure outlined above. Two sets of these data are reported here.

Nine shrubs (5 years old, dry-land culture) were harvested, defoliated manually, and separated into batches on the basis of gross anatomical differences, e.g., flower stems, leaves, wood (xylem), bark (phloem), etc. (figs. 1, 2). These fairly discrete parts were then cut by hand into small pieces, dried in a vacuum oven at 65° C. for 60 hours, processed in

TABLE 1

DISTRIBUTION OF WAX IN GUAYULE TISSUE DURING ACTIVE GROWING SEASON

Plant part	Tissue		Rubber in tissue (%)†	Resin in tissue (%)†	Wax	
	Total weight (gm.)*	Fraction of total (%)			In tissue (%)	Fraction of total (%)
Flower stems	87	2.28	0.51	7.26	0.52	2.24
Leaves	356	9.31	1.36	10.47	0.32	5.63
Tips:						
Bark (phloem)	338	8.84	15.66	11.25	1.69	28.26
Wood (xylem)	138	3.61	14.99	5.76	0.0	0.0
Stems:						
Bark	381	9.96	21.86	9.89	1.73	32.61
Wood	183	4.79	12.69	4.46	0.0	0.0
Branches (small):						
Bark	394	10.30	24.83	8.16	1.12	21.83
Wood	215	5.62	12.46	4.62	0.0	0.0
Branches (large):						
Bark	379	9.91	21.58	8.24	0.44	8.25
Wood	282	7.37	12.07	5.57	0.0	0.0
Crown:						
Bark	114	2.98	25.94	7.76	0.21	1.18
Wood	147	3.84	9.07	0.05	0.0	0.0
Root:						
Bark	196	5.13	25.64	10.27	0.0	0.0
Wood	353	9.23	0.90	9.94	0.0	0.0
Root (small):						
Unfractionated	261	6.83	16.13	10.45	0.0	0.0
Total, or weighted average	3824	100.00	14.82	8.49	0.53	100.00

* Moisture-free basis employed throughout.

† Analyzed by SPENCE-CALDWELL method (2)

a Wiley mill through a 5-mm. screen and in a hammermill. Samples were then taken for wax, resin (acetone extract), and rubber analyses, the latter two by the SPENCE-CALDWELL method (2). The results are presented in table 1.

Similar determinations made on shrubs of the same variety and age, except for growth period, but harvested during the dormant season, showed no pronounced changes in wax distribution (table 2).

Conclusions and summary

Thus it appears that guayule wax occurs almost exclusively in the phloem above the crown and in the actively growing parts of the shrub, where it probably protects these tissues from excessive dehydration during periods of moisture stress. Wax was also found in resinous guayule rubber to the extent of at least 0.5%. The effects of climatic and agronomic factors on wax distribution were not investigated beyond the evidence, outlined in table 2, which indicated that the wax distribution remains about constant for shrubs of the same age and variety harvested at different seasons.

TABLE 2

SEASONAL INFLUENCE ON CONTENT OF WAX IN GUAYULE SHRUBS

PLANT PART	WAX			
	Active growing season*		Dormant season†	
	In tissue (%)	Fraction of total (%)	In tissue (%)	Fraction of total (%)
Flower stems	0.52	2.24	0.35	2.58
Leaves	0.32	5.63	0.83	4.84
Tips, stem, and branch bark	1.19	90.95	0.85	90.71
Crown bark	0.21	1.18	0.23	1.86

* Harvested on April 21, 1948.

† Harvested on February 25, 1948.

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